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POTENTIAL VACCINE FOR ANTHRAX

ANNUAL\FINAL REPORT

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The main aim of this study has been to isolate, purify, and resolve the structure of the cell wall polysaccharide (pCHO) of Bacillus anthracis, and to study its properties and distribution on the cell as well as spore surfaces. The distributions and stability of PA (protective antigen) and EA (surface extractable antigen) were also studied using various methods.

In order to obtain the pCHO, B. anthracis delta-Sterne cells were grown overnight in Penassay broth and disrupted by sonication. The cell wall preparation was purified by sequential extraction with 1% hot SDS and several washes in water. Freeze dried cell wall (100 mg) was treated with 20 ml 50% HF at 4°C for 18 hr. The preparation was centrifuged and the supernatant was added to cold absolute ethanol in 1:5 ratio and allowed to stand 30 min at 4°C. The precipitate that was formed (pCHO) was separated

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19. ABSTRACT/SUMMARY (continued)

by centrifugation and washed 3 times with absolute ethanol. The pCHO was then dissolved in a ml of d-H_2O and freeze-dried for future analysis. (772)

Amino acid analyses of the pCHO revealed little or no muramic acid. The anthrone assay for neutral sugars gave a consistent hexose content per mg pCHO from one batch of the pCHO to another, suggesting the reproducibility of the method of purification.

Chemical analyses revealed the presence of galactose, N-acetylglucosamine, N-acetylmannosamine and galacturonic acid in molar ratios 2:1:1:0.2, respectively. However, gas chromatographic-mass spectra methods gave the molar ratios of 3:2:1:0.2. A TLC chromatogram of pCHO hydrolyzate confirmed the presence of galactose, glucosamine and mannosamine, but the absence of galacturonic acid. Sodium periodate oxidation of the pCHO destroyed approximately 93% of the galactose residues and 50% of the galacturonic acid residues. The two amino sugars were resistant. This implies that the galactose residues were either at the non-reducing end of the pCHO or the periodate-sensitive position within pCHO. Following sodium periodate oxidation and dialyses, the non-dialyzable fraction was reduced with sodium borodeuteride. The reduced product was reoxidized with sodium periodate and analyzed. All sugar components were recovered in the same molar ratio as was obtained following the first periodate oxidation. This implies that the pCHO moiety is made up of a linear backbone chain of galactose, glucosamine and mannosamine, probably linked by 1-->3 or 1-->4 glycosidic bonds. Amino sugars may be internally linked, since they were resistant to periodate treatment. As a side step, B. anthracis Sterne cells were treated with 0.2 M sodium periodate (4°C, overnight, covered) and employed in a lectin-cell aggregation assay using soy bean agglutinin (SBA). The untreated, Sterne cell[illegible] owed strong aggregation in the presence of SBA. Concentrations as low[illegible] ug/ml were able to bring about discernible aggregation. On the othe[illegible], the sodium periodate modified cells were totally resistant to SBA. [illegible]ent-rations as a high as 500 ug/ml failed to bring about aggreg[illegible]use SBA has specificity for galactose or N-acetylgalactosamine resid[illegible]; the sodium periodate does indeed destroy these moieties on the cells and renders them incapable of aggregation with the lectin.

When the Smith degradation product (SDP) was hydrolyzed with 0.1 M HCl for 3 hr at 100°C, glycerol and threitol were liberated. Glycerol is supposed to be liberated either from the non-reducing terminal galactose residue or from the internal galactose linked through positions 1 & 4. Because both glycerol and threitol were liberated from the SDP, some of the galactose residues were either at the non-reducing terminus or linked through positions 1 & 2 or 1 & 6, while some were linked by either 1,3 or 1,4 bonds. HPLC analyses revealed a 1:1 molar ratio of the glycerol and threitol. ^{13}C-NMR results showed that only two types of linkages, 1,4, & 1,6 were found in the HF-extracted pCHO. This observation thus eliminated the presence of either 1,2 or 1,3 bonds in the pCHO. ^{13}C-NMR studies showed the presence of two α and two β anomers. This agreed with the observations of the chromium trioxide oxidation which showed that the galactose residues were β-linked, while the amino sugars appeared to be α-linked. The most likely structure of the repeating unit of the pCHO is therefore:

->4(Galβ(1->6)Galβ(1->4)GlcNAcα(1->4)Galβ(1->4)
GlcNAcα/β(1->4)manNAcα(1->.

Monoclonal antibody (MAb) (obtained from the Army) raised against the Bacillus anthracis pCHO coupled with fluorescein-labelled anti mouse Ig was employed to detect the pCHO on the surface of the cell. All three strains of the vegetative cells showed fluorescence on the entire surface of the cell, while the spores were refractory. This suggests the presence of pCHO epitopes on the surface of B. anthracis cells and their absence on the surface of the spores. Electron microscope studies corroborated the fluorescence studies. When the cells were immunolabelled using the pCHO monoclonal antibodies, followed by gold-labelled anti Ig, the gold particles were seen adhering to the outer surfaces of the cells. This pCHO-MAb reaction is a very strong and stable one. When the organisms were boiled in order to determine the effect of heat on the pCHO, there was no decrease in the fluorescence. Instead it seemed to be more intense and diffusely spread over the entire surface of the cells. The electron micrographs also showed no decrease in the gold labelling. Possibly boiling exposed more pCHO epitopes on the vegetative cell surfaces due to heat removal of the cell-surface proteins, which may have masked some pCHO epitopes.

The spores were refractory to the polysaccharide-monoclonal antibody when studied by the fluorescence-antibody technique. The same organisms when sectioned for EM followed by immunolabelling directly on the grids, gave an interesting result. The gold particles were seen around the inner core of the spore, adjacent to the cortex. This is an appropriate finding, because it represents the region where the pCHO moiety is located. As the spore germinates into a new vegetative cell, components of the inner core region ultimately becomes a small part of the outermost covering of the cell. SDS and urea treatments of the cells gave observations similar to the heat-treated cells. The fluorescence was bright and diffusely distributed on the cells. When the vegetative cells were treated with the MAb to pCHO in the presence of pure pCHO (as a competitive inhibitor), the intensity of fluorescence was significantly reduced, proving that the MAbs provided by the Army and used in these studies, were indeed specific for the organism's pCHO.

Protective antigen (PA) was also shown to occupy the cell surfaces. Fluorescence-antibody studies using the PA-MAbs (Army) revealed the distribution of this protein on the complete surface of the cell. The fluorescence response however was not extremely bright and it disappeared totally when the cells were boiled or treated with SDS or urea. The corresponding EM studies revealed a random distribution of gold-particles around the cells.

The extractable antigen (EA) was also found on the cell surfaces. Fluorescence studies showed the distribution of EA on the whole surface of the vegetative cells. Intensity of fluorescence was weaker than that of the pCHO-MAb interactions. EM studies depicted a very well defined distribution of gold-particles all around the surface of the vegetative cells. Fluorescence became considerably faint when the cells were boiled before exposing to the EA-MAb. Some patchiness in fluorescence was observed following trypsin treatment. All fluorescence was lost when the cells were extracted with SDS or urea. EM studies corroborated the above findings. The cell extracts, when subjected to SDS-PAGE, revealed the presence of several protein bands. Western blots aided in identifying the bands that were specific for the various monoclones. The protein extracted

from the cells also gave a positive color reaction when employed in the ELISA technique in conjunction with the EA-MAb.

Treating the B. anthracis cells and spores with fluorescein-labelled SBA showed that both the vegetative cells and spores are bound by the lectin, hence fluorescence was observed throughout the entire surface of the cells and spores. The lectin reactive sites on the spore surface may be limited compared to the lectin receptors on the vegetative cells, hence the spores showed a much weaker fluorescence. When the heat-modified vegetative cells or spores were treated with fluorescein-labelled SBA, the vegetative cells and spores showed enhanced fluorescence. This shows that the lectin receptors are non-protein in nature (galactose is a neutral sugar), and thus are not affected by heat. The lectin-reactive sites may be exposed more, due to the inactivation of surface proteins by heat and this brings about an unmasking of more lectin receptors on the surface and hence greater fluorescence.

In an independent experiment, the SBA was acetylated using ^{3}H-acetic anhydride and then used with the various strains of B. anthracis cells, cell walls, spores as well as trypsinized and heat-treated cells. The spores appeared to have more bound SBA than the corresponding cells. The cell walls showed maximum uptake of the lectin. Cells that were trypsinized or heat-modified showed an increase in the amount of bound SBA as compared to the untreated cells.

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FLUORESCEIN LABELLED INDIRECT ANTIBODY STUDIES

AIM: - To study the surface of B. anthracis cells and spores using monoclonal antibodies to polysaccharide (pCHO), protective antigen (PA) and the surface extractable antigens (EA). The pCHO and EA monoclonal antibodies were obtained from Dr Ezzell and the PA MAbs were obtained from Mr. Steve Little, USAMRIID, Ft. Detrick, MD.

MATERIALS AND METHODS: - B. anthracis 4229, Sterne and Δ-Sterne strains were used for this study. The cells were grown overnight in Ristroph's medium (See section- "Protocols"). The cells were washed and suspended in 10 mM sodium phosphate, 0.85% sodium-chloride, pH 7.3 (PBS). This suspension was then diluted 2 fold into a series of 10 tubes. A 5 ul volume of each dilution was placed per well of a teflon- coated multi-well slide (PGC Scientific). In order to determine the best dilution, the slides were air dried, fixed in methanol and gram stained. The dilution which gave 5 to 10 cells per field was selected for future use. In some instances, the slides were prepared ahead of time and frozen.

For immunolabelling, the slides were washed 2 times with PBST (PBS + 0.6% Tween 20) and blocked for 30 min with PBSTG (PBS + 0.6% Tween 20 + 1% gelatin) at 37°C. Slides were processed in a closed vessel containing wet paper towels in order to prevent drying. Excess PBSTG was vacuumed off. 25 ul of the desired monoclonal antibody was diluted in PBSTG and placed on the wells for 1 hr at 37°C. For antibodies in tissue culture fluid, the dilution was 1:1 in PBSTG, and for antibodies in ascites fluid or sera, the dilution was 1:200. The wells were then washed 4 times with PBST, and then incubated for 30 min at 37°C with 40 ul of a fluorescein labelled goat anti-mouse Ig (IgG, IgA, IgM/FITC from Cappell), diluted 1:200 in PBSTG. They were then washed with PBST and mounted with a drop of DABCO mountant (1,4 diazobicyclo-(2,2,2)-octane, Sigma, at 25 mg/ml in 10% PBS-90% glycerol, pH 7.4). The slides had to be protected from light henceforth.

Several variables were introduced into these fluorescence studies. The 3 strains of B. anthracis cells were subjected to extractions with various concentrations of urea (at room temp or 70°C), NaSCN, 1% SDS/5mM mercaptoethanol (at 70°C, 30 min). Other treatments included boiling for 15 min or treating with trypsin.

The cells were studied using the MAbs against PA, EA & pCHO in conjunction with fluorescein labelled anti-Ig.

Earlier studies from this lab (1) have shown the agglutinability of B. anthracis with the soy bean aglutinin (SBA). SBA is a lectin, showing a specificity for N-acetyl-galactosamine and D-galactose residues. Cells and

spores were incubated with FITC labelled SBA in order to study the distribution of these sugars on the surfaces of the organism.

Cells were blocked with MAbs to pCHO, followed by anti-EA or anti-PA. In other instances, the pCHO-monoclone binding sites were blocked with the surface array proteins EA-I & EA-II followed by the anti-pCHO. Several similar blocking regimens were also tried before exposure to FITC-SBA.

The spores of B. anthracis were grown in Leighton-Doi modified medium (See Section "Protocols"). Multi-well slides were prepared in the same manner as the cells. The spores were studied using FITC labelled SBA, MAbs to pCHO, PA and EA. The spores were also boiled for 15 min and subjected to the same treatments.

RESULTS AND DISCUSSION : -

(i) B. anthracis cells with MAbs to pCHO: All three strains of the bacterium fluoresced very brightly when exposed to the monoclonal Abs against pCHO. The fluorescence was very uniformly and evenly distributed on the surface of the cells. It had a translucent nature with distinct boundaries (Figs 1 thru 4), suggesting that the pCHO structure is evenly distributed throughout the surface of the cells. The 4229 cells seemed to fluoresce somewhat brighter than either Sterne or Δ-Sterne (Fig 5&6). This fluorescence is very stable and consistent. An interesting phenomenon was seen however when the cells were boiled before exposing to the MAb to pCHO. The fluorescence appeared to be more intense, somewhat diffuse and opaque and covered the whole cell surface like a thick, glowing mat (Figs 7&8). Treatment of the cells with any of the mentioned denaturants, brought no loss in the fluorescence intensity (Figs 9 thru 12). Extracting the cells with 1% SDS (37°C, 1 hr) did not eliminate their ability to fluoresce with the MAbs to pCHO.

(ii) B. anthracis cells with MAbs to PA: Fluorescence studies revealed a uniform, surface distribution of the protective antigen. The intensity of fluorescence was considerably lower than that observed with the anti-pCHO (Figs 13 thru 16). This fluorescence was readily lost if the cells were pre-treated with SDS or urea. The cells then acquired an overall yellowish hue, which resembled the untreated, control cells. When the cells were treated with trypsin (100 ug/ml, 1 hr, 37°C), the fluorescence displayed distinct patterns of granularity. Preincubation of the cells with SBA seemed to bring about a patchiness in the fluorescence distribution.

(iii) B. anthracis cells with MAbs to EA: The fluorescence was uniformly distributed over the entire surface of the cells. The intensity was markedly reduced as compared to the anti-pCHO studies. The cells that were grown in R-medium overnight (instead of agar plates) displayed a unique pattern of fluorescence distribution (Figs 17 thru 21). Distinct, bright patches of fluorescence were obvious on the edges of the cells. When the cells were extracted with 6 M urea, prior to MAb exposure, most of the patches either disappeared or became very faint (Fig 24). Treatment of the cells with the SDS/mercaptoethanol mixture (70 ^{o}C, 30 min) rendered them totally nonfluorescent (Figs 22&23). Boiling of the cells for 15 min made them non-fluorescent. It was impossible to photograph these faint cells.

Thus it appears that it is quite easy to remove this entity from the surface of the cells. Protein extractions as well as EM studies (other sections of the report) have shed further light to support the findings on the distribution of pCHO, PA and EA.

(iv) B. anthracis Spores: The spores of this bacterium did not fluoresce when subjected to the indirect-Ab studies using anti-pCHO, PA or EA. Δ-Sterne spores fluoresced brightly however when treated with anti PA (Fig 28). This was difficult to explain. The spores did fluoresce with the FITC labelled lectin, soy bean agglutinin (SBA) (Fig 25). The boundaries of the spores displayed a more distinct fluorescence, the intensity of which increased somewhat when the spores were boiled for 15 to 30 min before treating with the FITC/SBA (Figs 26&27).

(v) Blocking of the pCHO-monoclone binding sites with surface array proteins EA-I, EA-II had no effects on fluorescence. The results obtained were the same as when the cells were not treated with surface array proteins. It appears that the pCHO structure is evenly distributed throughout the surface of B. anthracis.

(vi) Fig 29 shows fluorescing B. anthracis Sterne cells, after being treated with FITC-SBA. Some interesting patchy patterns of fluorescence distribution are obvious.

(vii) When B. anthracis Sterne cells were blocked with SBA before treating with 6G6-2-3, an anti pChO, the cells did not lose their ability to fluoresce (Figs 30&31).

Fig. 1
B.anthracis Sterne cells suspended in PBS and treated with FITC-Ig. No prior treatment with denaturants or monoclonal antibodies. These cells served as the controls. No fluorescence was discernable.

Fig. 2
B.anthracis Sterne cells exposed to 6G6-2-3 MAb to pCHO.
Note: Intense, uniform, fluorescence.

Fig.3
B.anthracis Sterne cells (in PBS), not treated with any denaturants. Processed with MAb to pCHO (6G6-2-3). The fluorescence is bright and translucent.

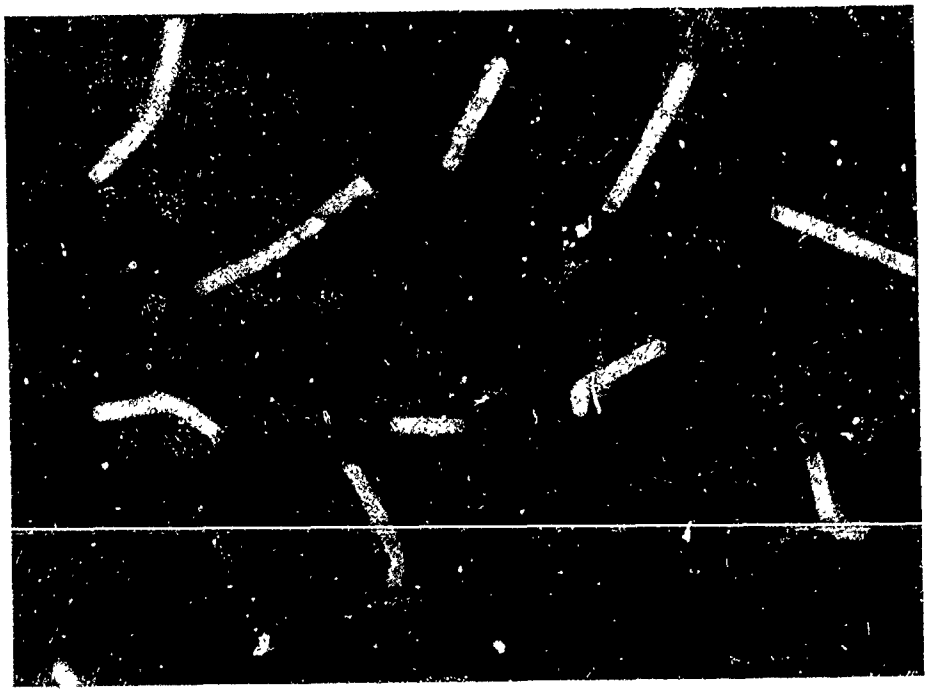

Fig.4
B.anthracis Sterne cells treated with MAb to pCHO (6G6-2-3) followed by FITC-antiIg. Notice the bright and overall fluorescence.

Fig. 5
B.anthracis 4229 cells (in PBS) not treated with any denaturants. Note: The uniform, overall, translucent nature of the fluorescence when exposed to the MAb to pCHO (6G6-2-3).

Fig. 6
B.anthracis 4229 cells in PBS, treated with the MAb to pCHO (6G6-2-3). Note: Very bright, overall fluorescence.

Fig. 7
B.anthracis Sterne cells boiled for 15 min before exposing to 6G6-2-3 MAb to pCHO.

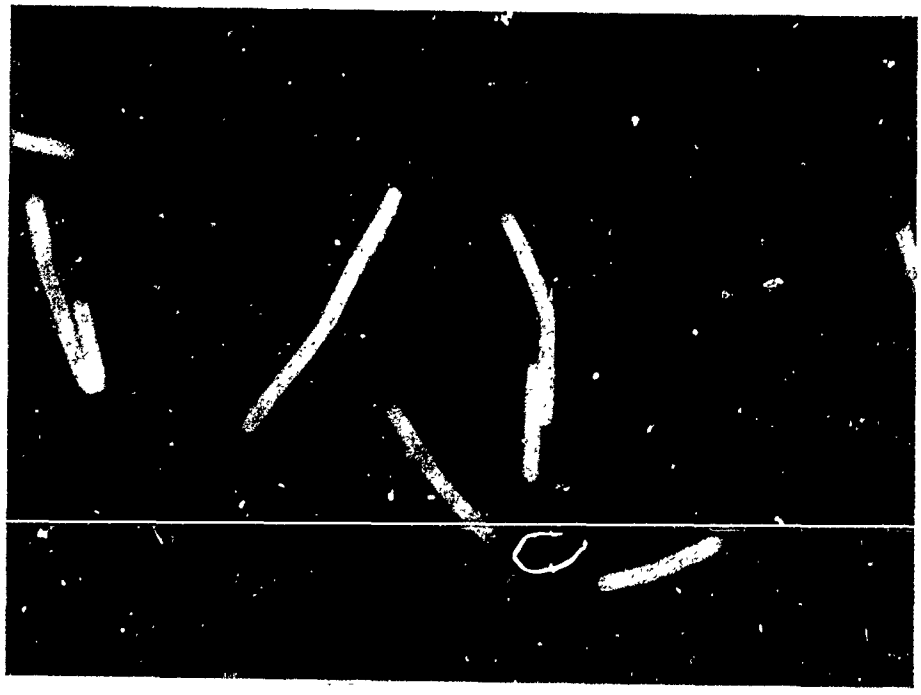

Fig. 8
B.anthracis Sterne cells boiled prior to treatment with the MAb to pCHO (6G6-2-3). Note: The fluorescence did not diminish, instead it attained a diffused and brighter hue.

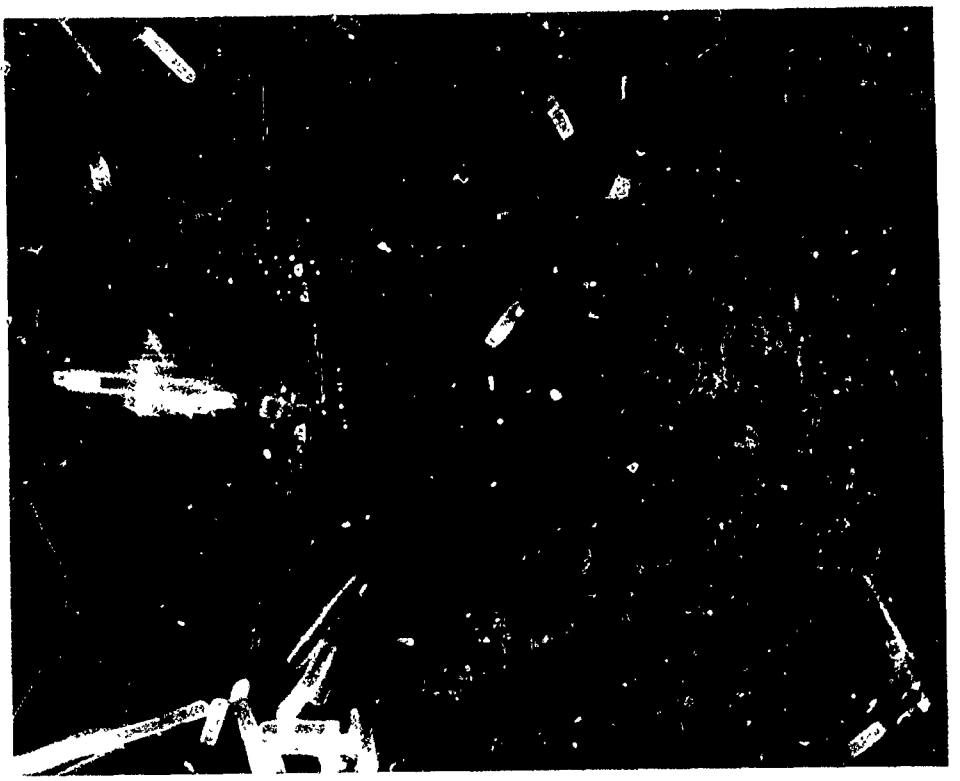

Fig.9
B. anthracis Sterne cells, grown in R-medium treated with 1% SDS (1hr,RT) followed by the MAb to pCHO (6G6-2-3). The treatment with the denaturant does not affect the distribution or intensity of the fluorescence in anyway. Note: The translucent nature of the fluorescence.

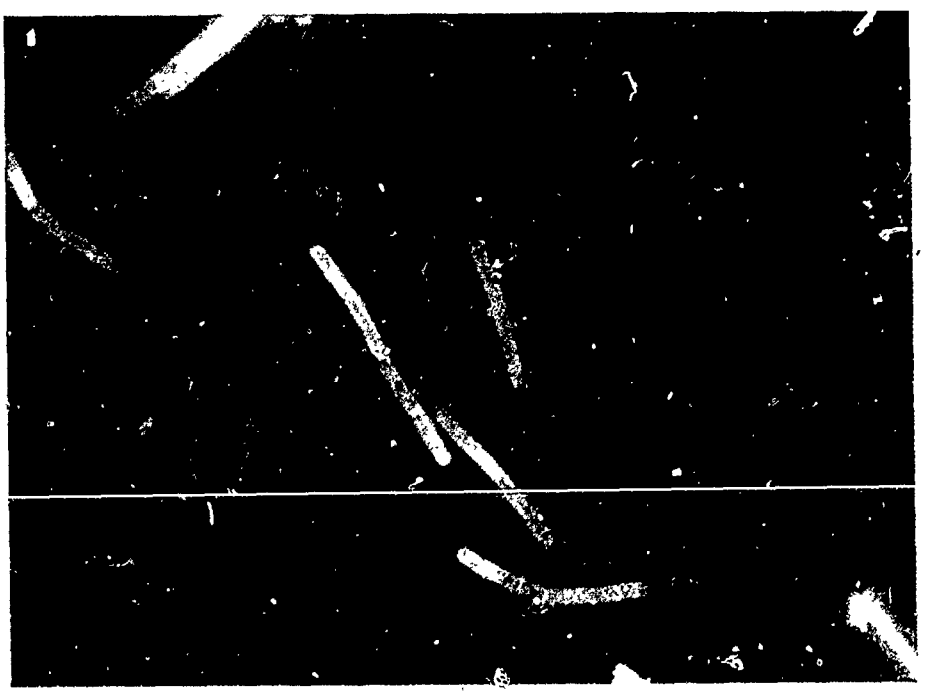

Fig.10
B.anthracis Sterne cells trypsinized prior to treatment with the MAb to pCHO (6G6-2-3). There was no loss in the intensity of fluorescence.

Fig. 11
B.anthracis Sterne cells treated with 1% SDS (1hr,RT) before exposing to the MAb to pCHO. The fluorescence seems brighter, smooth and more evenly distributed throughout the surface of the cell, when tested with the monoclonal antibody to pCHO.

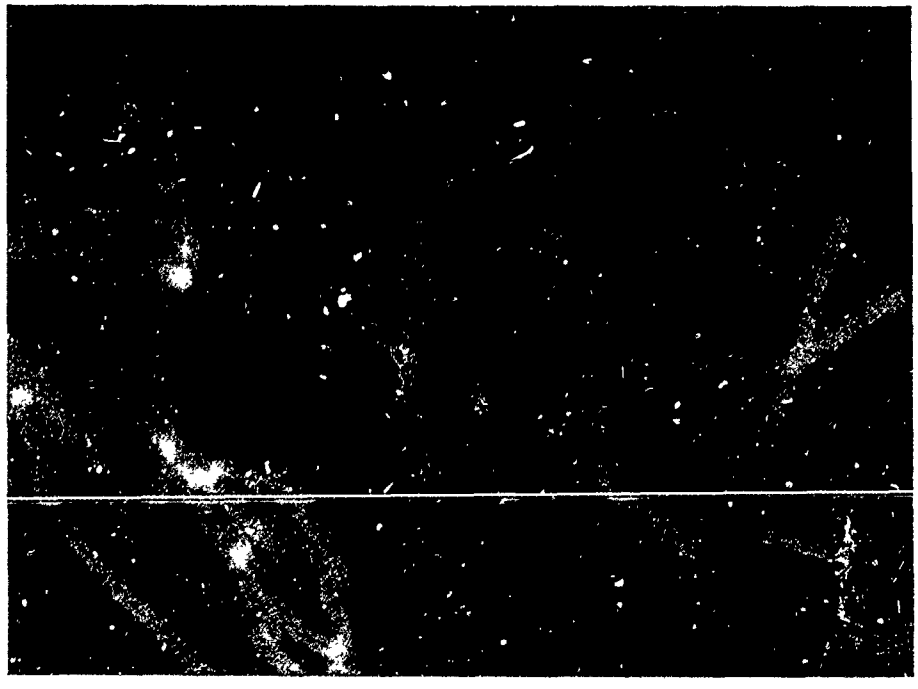

Fig. 12
B.anthracis Sterne cells pretreated with 1% SDS prior to exposure to antipCHO (6G6-2-3). Note: The fluorescence seems to be stronger and more diffusely spread on the whole surface of the cell.

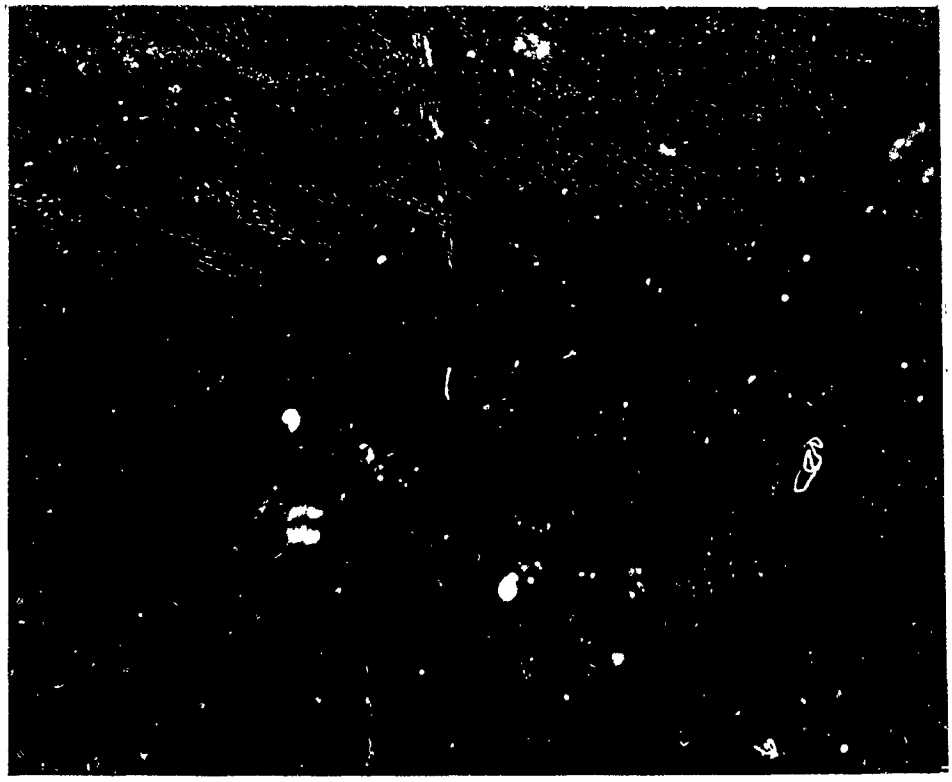

Fig. 13
B.anthracis Sterne cells in PBS, exposed to the MAb to PA (PA3 3C5-1-1). Cells do fluoresce, but not as bright as with pCHO-MAb. Overall granular distribution of the fluorescence is obvious.

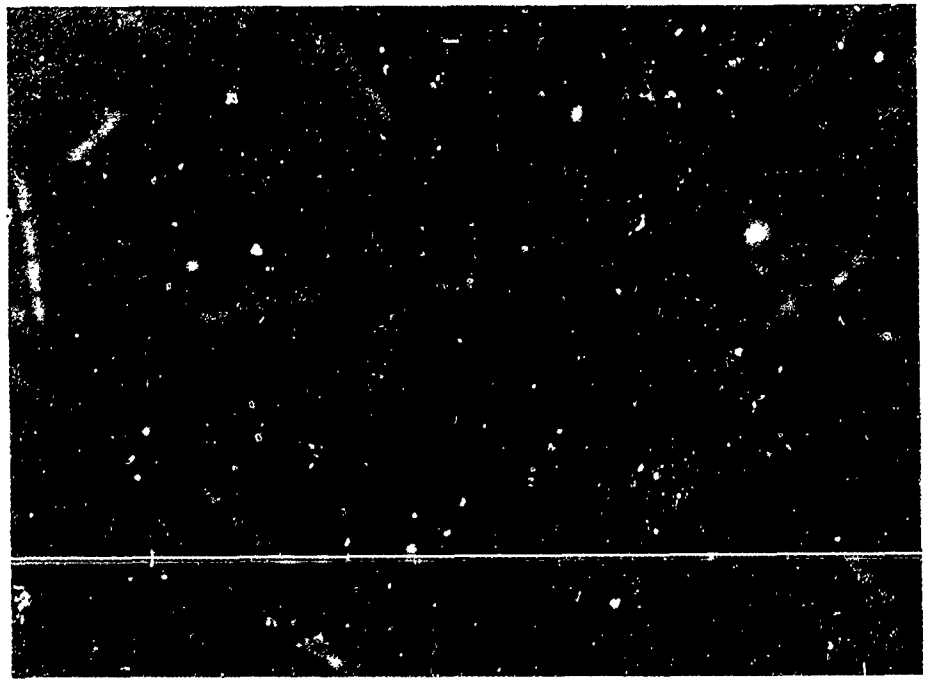

Fig. 14
B.anthracis Sterne cells treated with the MAb to PA (PA3 3C5-1-1),
followed by FITC-antiIg. Cells show fluorescence on the whole surface, implying the presence of a specific PA moeity on the surface of the cells.

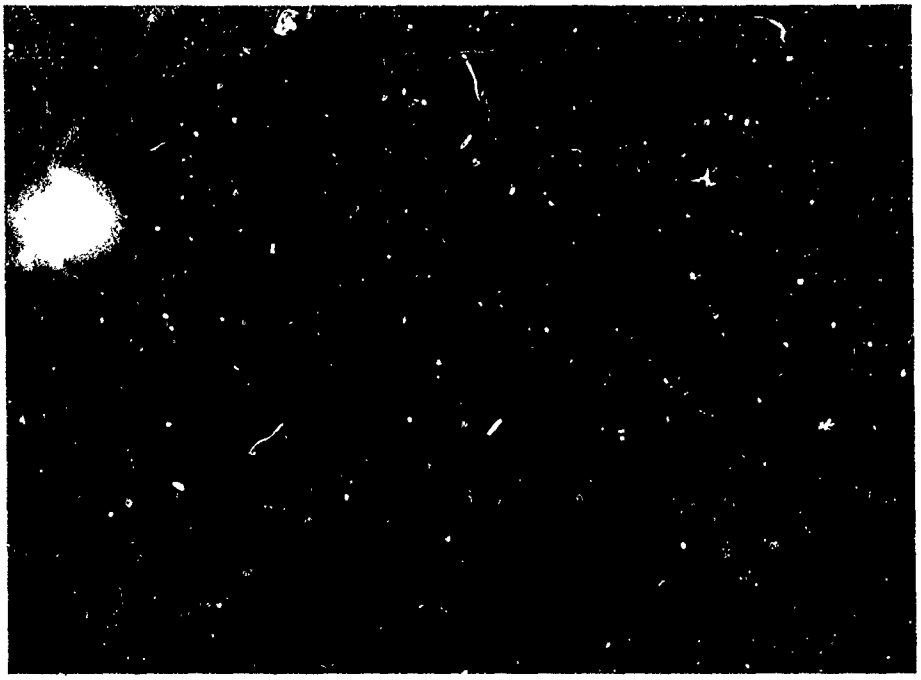

Fig. 15
B. anthracis 4229 cells exposed to MAb to PA (PA3-3C5-1-1). Note: The bright, overall, smooth fluorescence pattern.

Fig. 16
B.anthracis 4229 cells in conjunction with the MAb to PA (PA1-1G7-2-1).

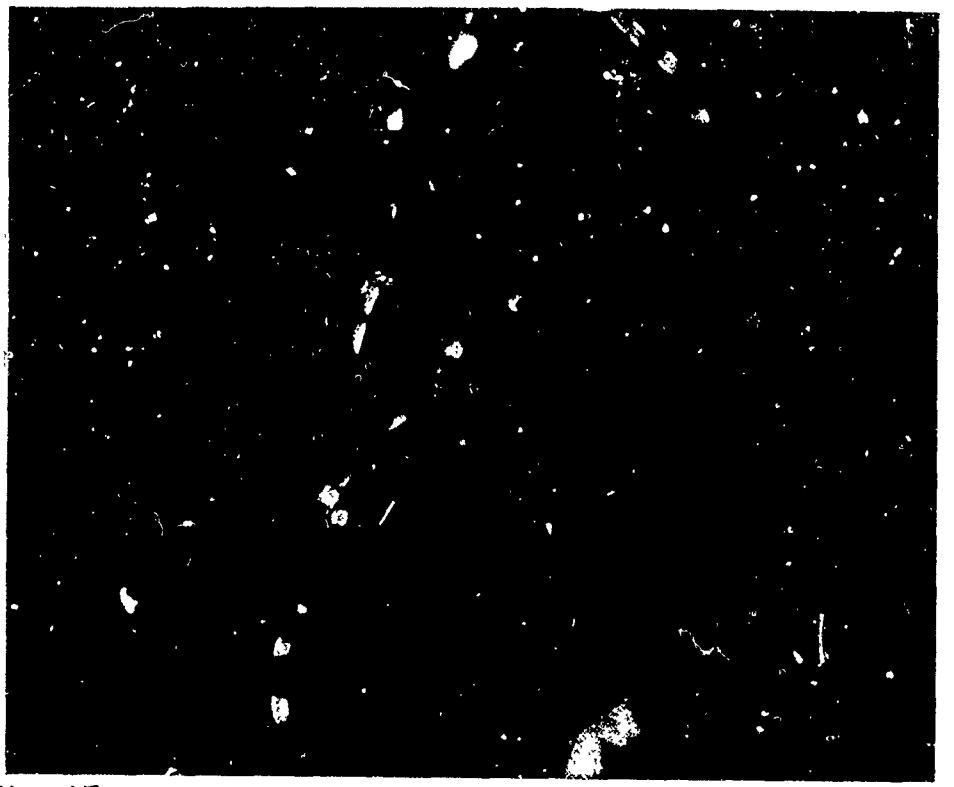

Fig. 17
B.anthracis Sterne cells grown overnight in R-medium. The cells were not exposed to any denaturants before exposing to EAII-2C2-1-1 MAb to EA. Notice the bright patches of fluorescence distributed intermittently on the surface of the cells.

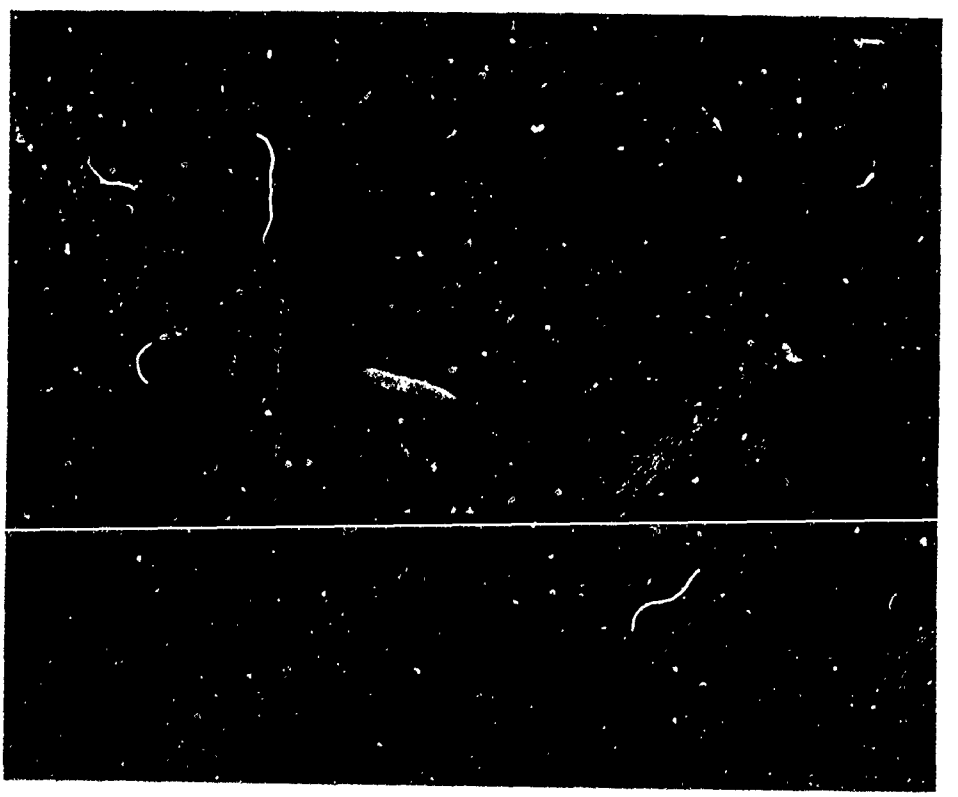

Fig. 18
Same as Fig. 17 above. The pattern of fluorescence distribution varies somewhat.

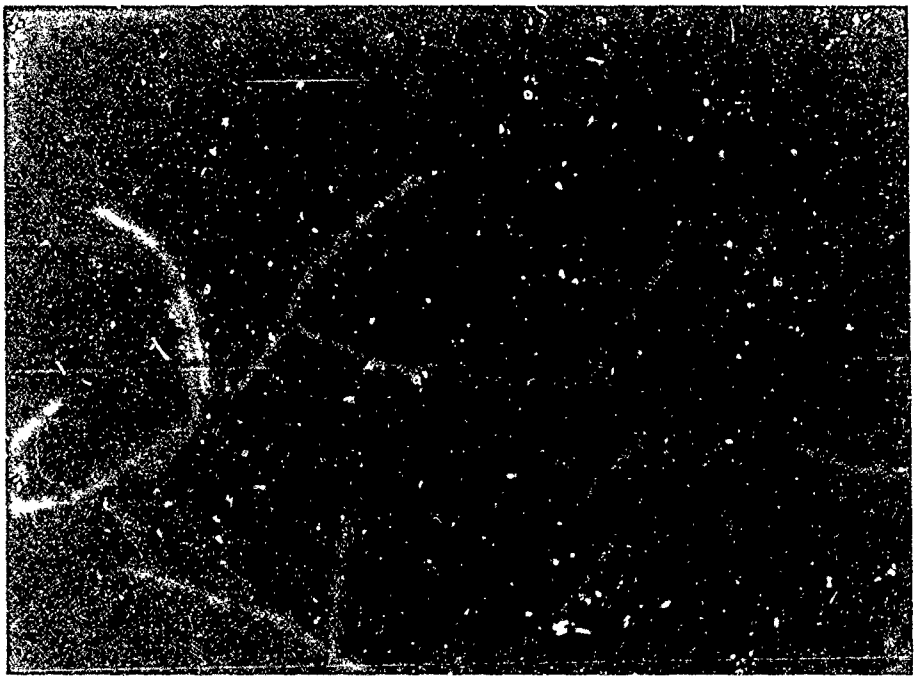

Fig. 19
B.anthracis 4229 cells exposed to the MAb to EA (EAII69). Cells display positive fluorescence, but significantly fainter than that seen with antipCHO.

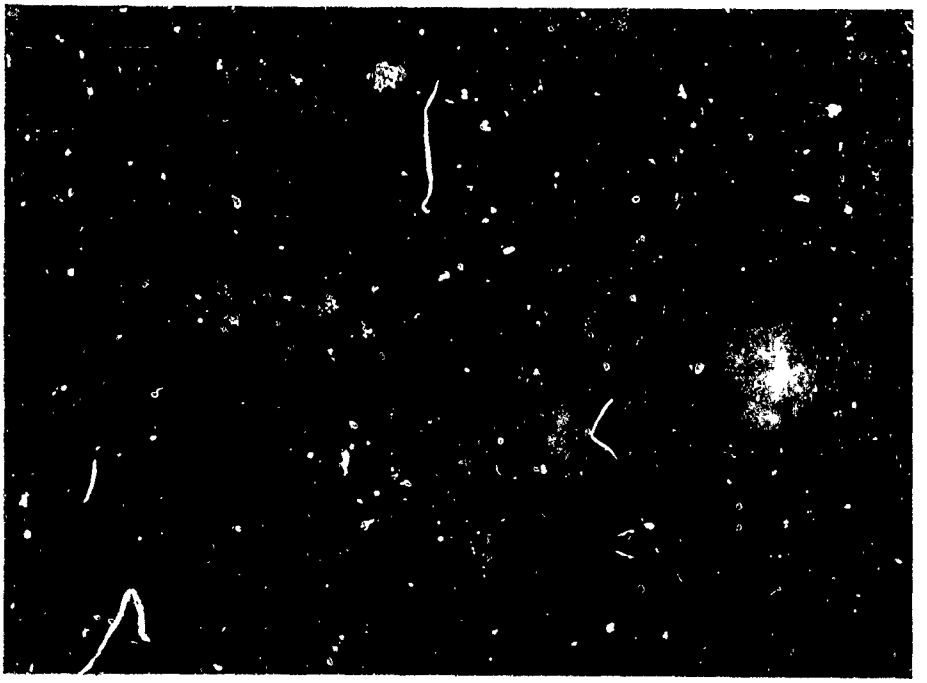

Fig. 20
A mixture of Δ-Sterne cells and spores exposed to the MAb to EA (1EAII 2G9). The cells fluoresce as seen earlier and as expected, but no fluorescence was discernible on the spores.

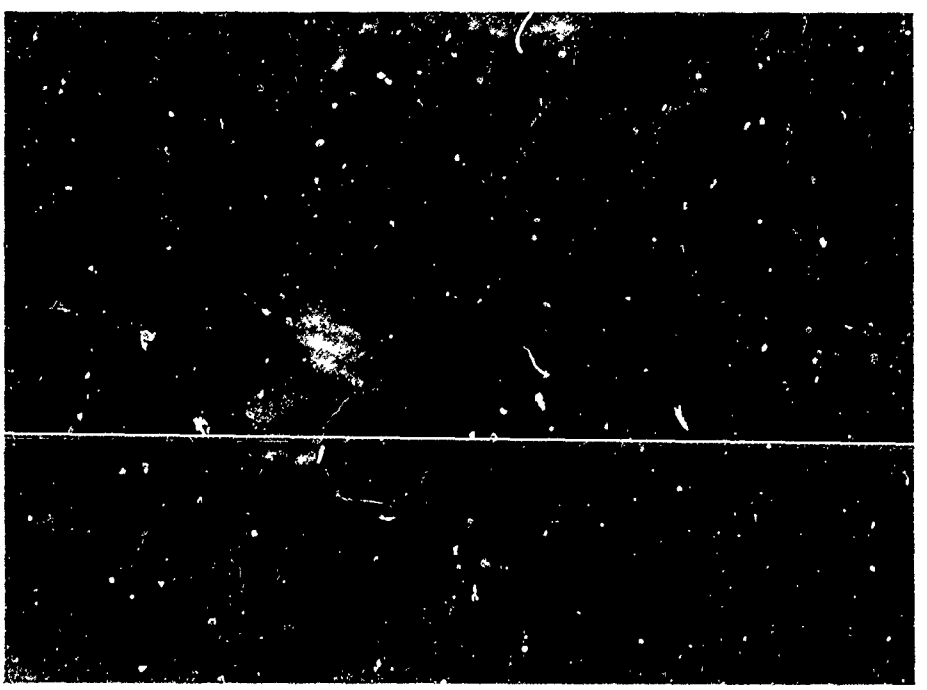

Fig. 21
B.anthracis Δ-Sterne cells exposed to MAb to EA (EAII 2G9). Note: Very irregular, patchy sort of fluorescence.

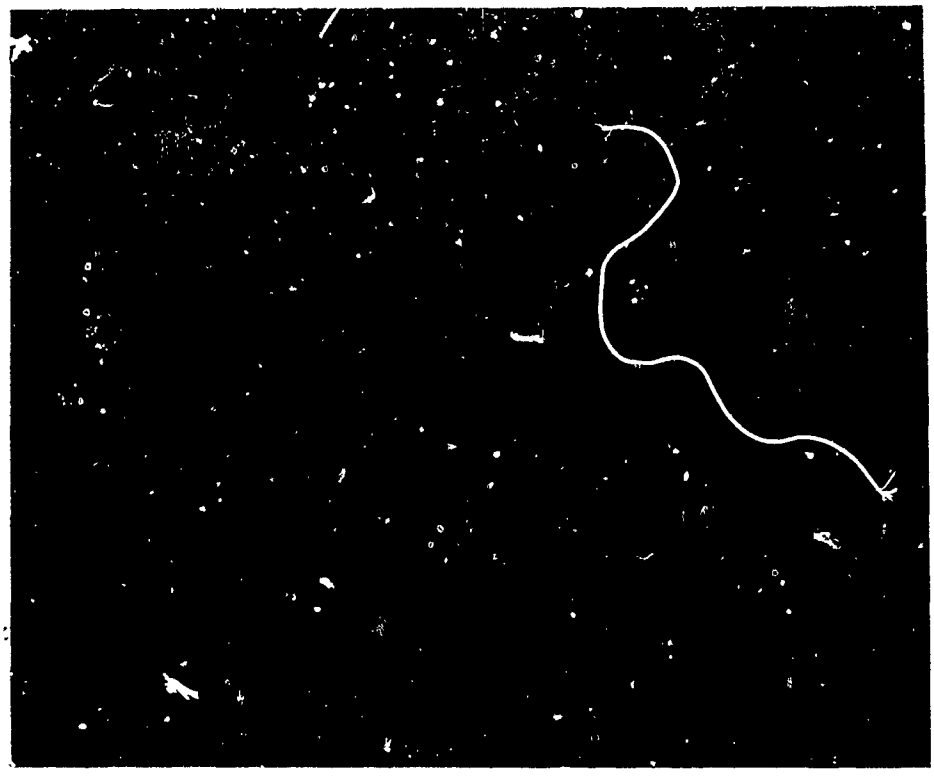

Fig. 22
B.anthracis Sterne cells grown overnight in R-medium. The cells were pretreated with 1% SDS (1hr,RT) before exposing to the MAb to EA (1EA-II 1D3-1-2). The intensity of fluorescence is greatly reduced, suggesting a loss of surface components specific for the monoclonal antibody.

Fig. 23
B.anthracis Sterne cells grown overnight in R-medium. The cells were pretreated with 1% SDS (1hr,RT) then exposed to 1EA-II 2C2-1-1 MAb to EA, followed by FITC-Ig. Note: The diminished, faint and patchy fluorescence

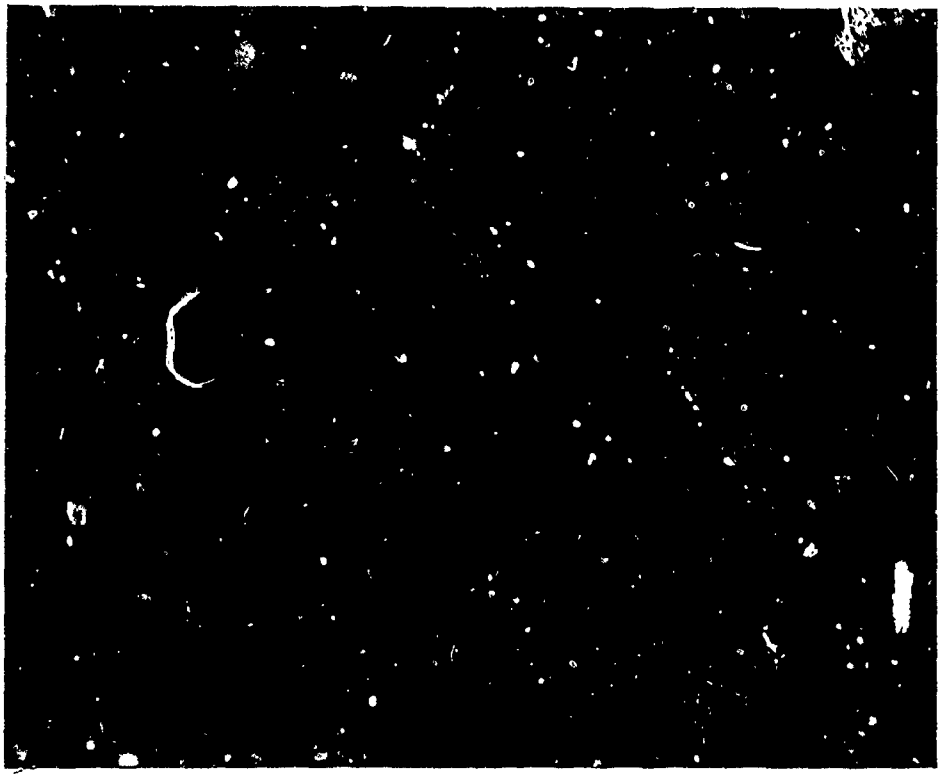

Fig. 24
B.anthracis Sterne cells grown overnight in R-medium and treated with 6M Urea (1hr,RT) before exposing to the MAb 1EAII-1D3-1-2. The fluorescence is present as fine granules or specks along the edges of the cells. The wide and bright patches of fluorescence are lost.

Fig. 25. B.anthracis Sterne spores treated with FITC-SBA. Notice the faint yet positive fluorescence.

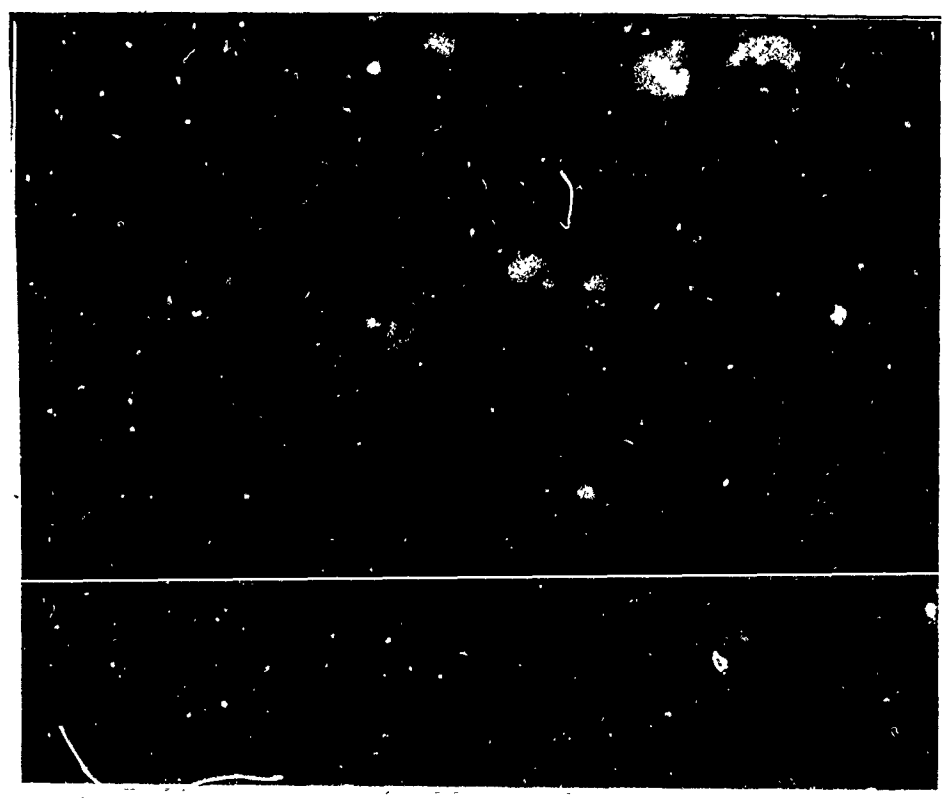

Fig. 26
B.anthracis Sterne spores boiled for 15 min before treating with FITC-SBA. The fluorescence appears to be more intense.

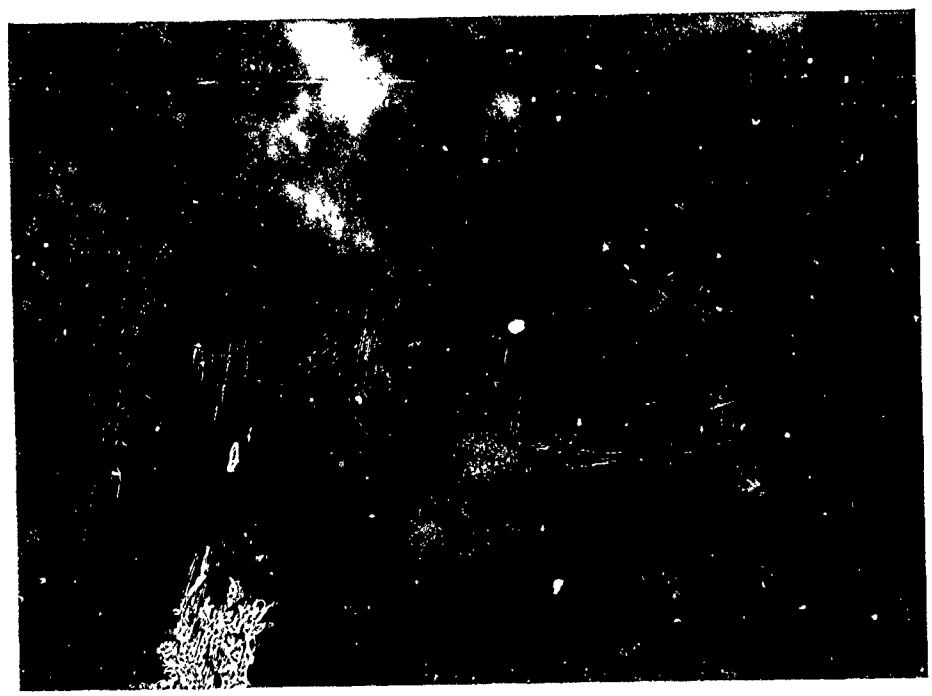

Fig. 27
B.anthracis 4229 spores treated with fluorescein labelled SBA.

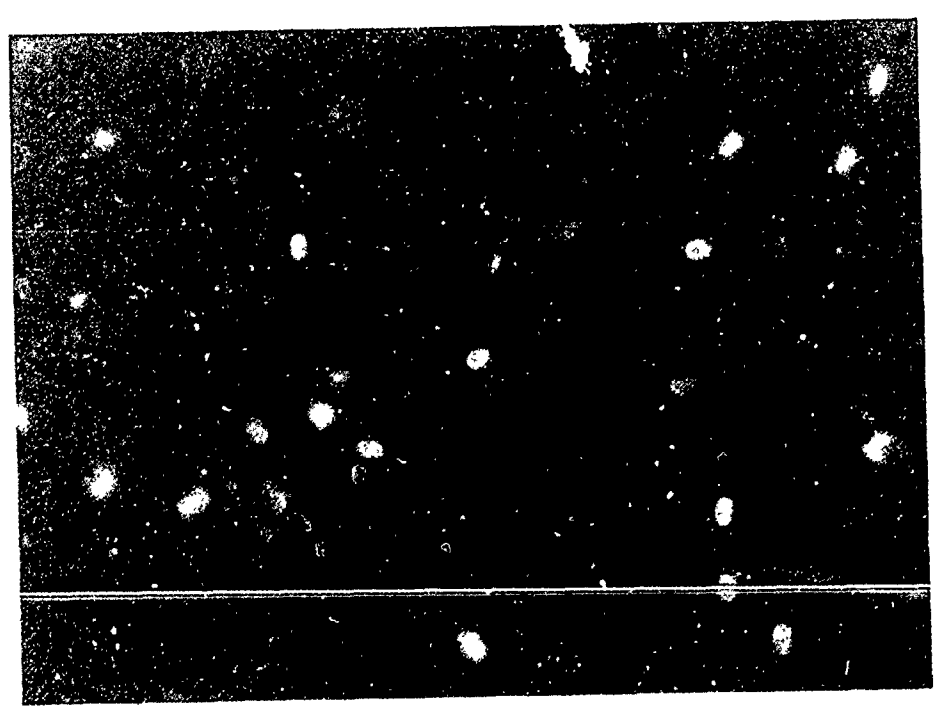

Fig. 28
Δ-Sterne spores treated with PA1-1G7-2-1 followed by FITC-antiIg. Note:The spores fluoresce very brightly, a rather perplexing observation.

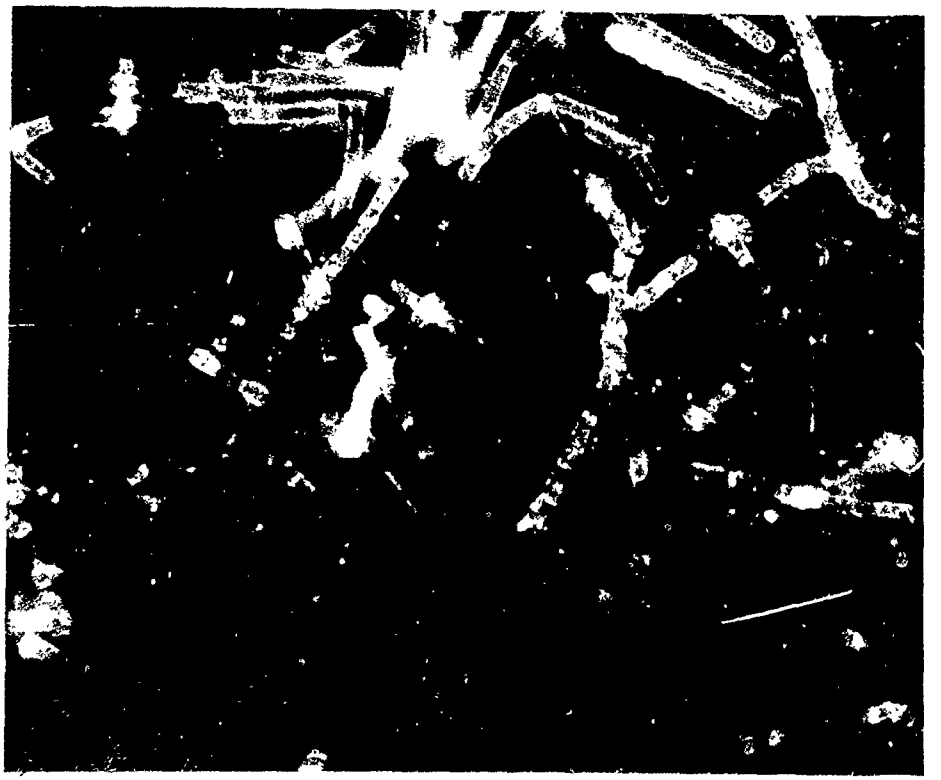

Fig. 29
B.anthracis Sterne cells grown in R-medium and incubated with SBA. The lectin seeks out galactose and N-acetyl galactosamine on the surface of the cells. Notice the strong fluorescence response.

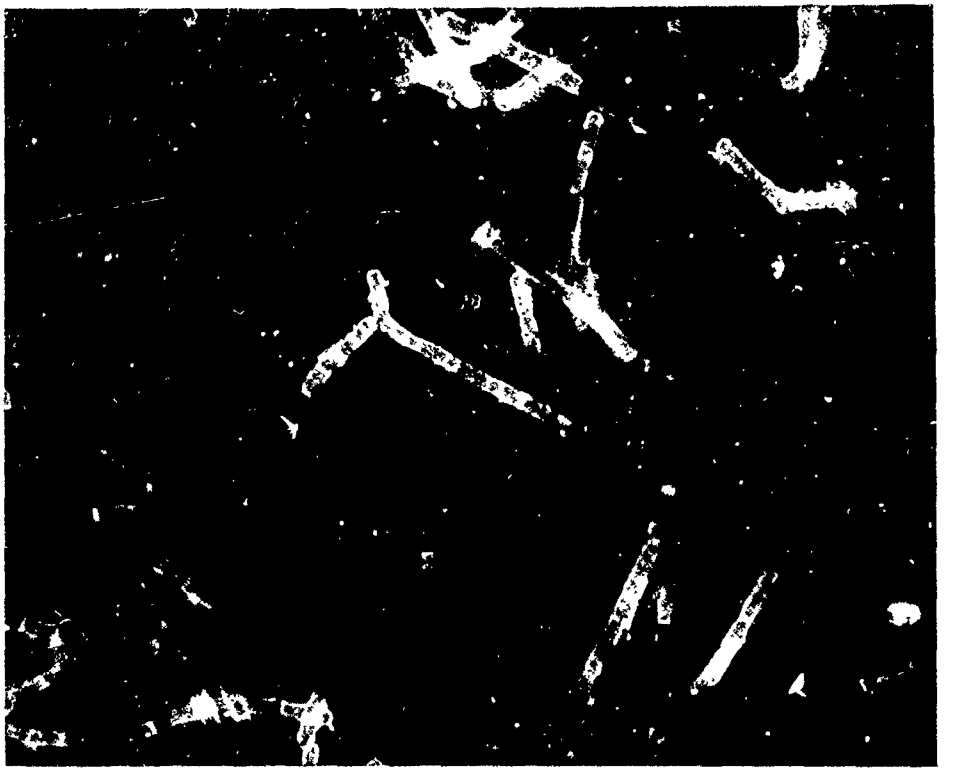

Fig.30
B.anthracis Sterne cells blocked with SBA (1hr,RT) before exposing to the MAb to pCHO (6G6-2-3). The fluorescence is strong, but displays a blotchy or irregular distribution.

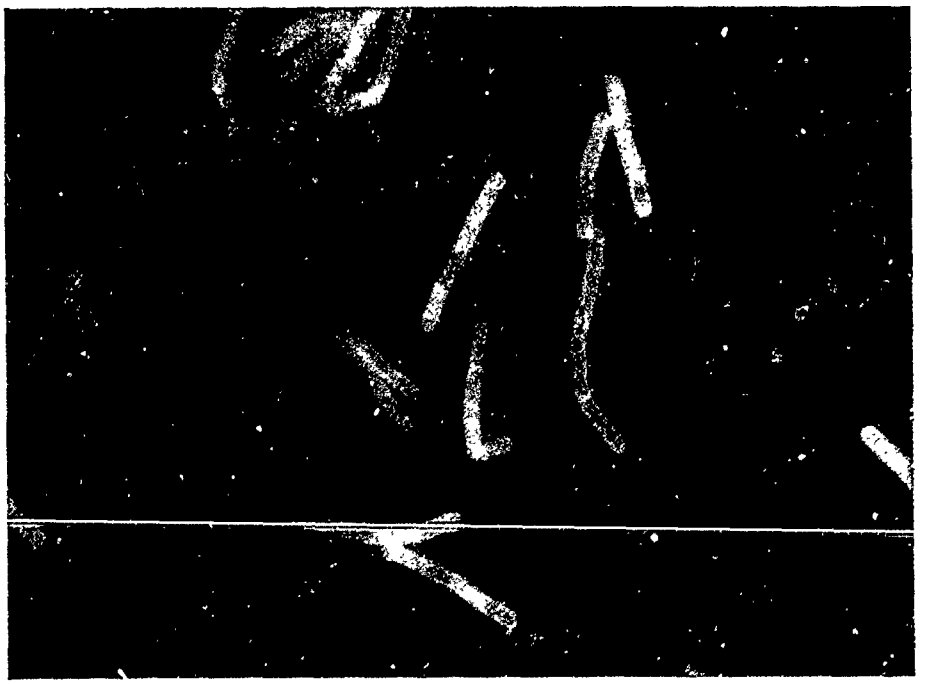

Fig.31
B.anthracis Sterne cells blocked with SBA prior to the antipCHO (6G6-2-3). The fluorescence is bright and comparable to the cells that were in PBS and not pretreated with any denaturants.

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Aim:-

To determine whether the monoclonal antibodies to pCHO provided by the Army, are actually against the pCHO moeity of the B. anthracis Sterne cells.

Method:-

Pure polysaccharide was isolated from the cell walls of B. anthracis Δ-Sterne cells. (See section "Protocols). One milligram of the purified pCHO was dissolved in 1 ml of a 1:200 diluted solution of PBSTG containing the monoclonal antibody against pCHO (6G6-2-1). 25 ul of the above solution were placed on the wells of a spot well slide containing Sterne cells. The control wells contained the MAb solution without the purified pCHO. The slides were incubated in a moist, closed chamber for 60 min at 37°C. After the recommended washings with PBST, the cells were incubated for 30 min with 40 ul of the FITC labelled anti Ig, diluted 1:200 in PBSTG. The slides were washed again and mounted with the DABCO medium.

Results:-

There was a significant decrease in the intensity of fluorescence for the cells that were exposed to the MAbs in the presence of purified pCHO. Conversely, cells that were exposed only to the MAbs, fluoresced extremely bright.

It is evident, that the pure pCHO in our cell-MAb system, served as a competitive inhibitor and due to its apparent specificity to the MAb, it bound readily to it, and thus making less of it available for the cell surface reaction.

It can be confidently stated at this point that the provided MAb to pCHO is in fact specific to the purified pCHO.

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ELECTRON MICROSCOPE STUDIES

AIM: - To perform EM studies in order to corroborate the findings of the indirect fluorescent antibody assays.

Several different processing protocols were tried until an ideal system was worked out. Emphasis and details will be given only of the successful method. It is desirable however, to briefly mention the methods that did not work. (i) The cells and spores of B. anthracis were grown in R-medium and Leighton Doi Medium respectively, washed and suspended in appropriate buffers and immunolabelled in Eppendorf tubes using the various MAbs. Following the initial incubation, samples were washed and treated with gold labelled anti-Ig (Sigma). These organisms were then subjected to dehydration, embedding (in Epon 812) and cutting of the blocks. The sections were then stained with uranyl acetate and lead citrate. The results were not satisfactory. The labelling was sparse and usually nonspecific. (ii) The second attempt involved the use of LR-white (Ladd Research Industries, Inc.) as the embedding resin instead of Epon 812. LR-white is supplied as a blend of hydrophilic acrylic monomers which exhibit extremely low viscosity for rapid and thorough penetration. Because of this special hydrophilic nature, immunochemicals and antibodies are shown to easily permeate the LR-resin and reach their complementary sites. The resin has low toxicity, requires no hazardous mixing and can be used directly from the alcohol dehydrant. Epon 812 on the other hand requires a rigorous alcohol series dehydration followed by curing at 60°C for 48 hr or more. LR-white can be used at ambient temperatures with an accelerator or heat cured at 50°C for 24 hr. We opted for the latter, because it gives more control of the curing process. (iii) An ideal system was achieved when the organisms were sectioned first and the immunolabelling thereafter, directly on the grids. The Au-labelling became significant and specific.

MATERIALS AND METHODS:-

Protocol for Embedding and Immunolabelling:

The cells were grown in R-medium and the spores in Leighton Doi spore medium. After appropriate washings, the organisms were placed in 70% alcohol for 10 min. The cell and spore pellets were placed in Eppendorf tubes and processed as followed:

70% alcohol: LR-white (2:1) -- 4 hr
70% alcohol: LR-white (1:1) -- Overnight
70% alcohol: LR-white (1:2) -- All day
70% alcohol: LR-white (0:1) -- Overnight
70% alcohol: LR-white (0:1) -- All day

The pellets were then carefully transferred to gelatin capsules containing LR-white and allowed to cure overnight at 50°C. This formed a hard block. The gelatin could be soaked or peeled away. The block was trimmed and sectioned. The sections were picked up on 200 mesh nickel grids which were pretreated with Formvar and also carbon coated for greater stability.

For immunolabelling, the grids were blocked by floating them on 25 ul drops of a 50 mM TRIS Buffer, pH 8.2 [Tris (hydroxy methyl) aminomethane], containing 1% BSA for 15 min. They were then floated (section side drown) on a 25 ul drop of the desired MAb, diluted in TRIS-Buffer for 1 hr at RT. The grids were grasped with a fine pair of forceps and washed with TRIS-Buffer. They were then floated on 40 ul drops of Au-anti-Ig (A colloidal gold mixture of IgG, IgM and IgA labelled with 20 nm size gold particles, EY Labs) and incubated for another hr at RT. The grids were given a final wash with TRIS, devoid of the BSA. It was necessary to eliminate all traces of BSA in order to avoid interference with the glutaraldehyde fixation and nonspecificity later on. The grids were fixed in 2% glutaraldehyde for 2 min, washed with water and then stained for 30 sec in 5% uranyl acetate followed by 30 sec in lead citrate (procedure in "Protocols" section). After final washings with water, the grids were ready for transmission electron microscopy.

RESULTS AND DISCUSSION:-

(i) Vegetative cells treated with MAbs to pCHO (6G6-2-1 and 5G4-2-3):

Figs. 32 and 33 represents control cells which were not treated with any MAbs, only Au-labelled anti-Ig.

All three strains of B. anthracis showed significant gold labelling on the surface of the cells. The particles were uniformly distributed around the cells. There was no intracellular labelling. This was indicative of the presence of a continuous pCHO layer around the cells and is also a confirmation of our corresponding FITC studies. The cells of the 4229 strain appeared to lodge a greater number of Au-particles on their periphery (Figs 34 thru 38), this was again in agreement with the previous fluorescence studies, that showed a stronger fluorescence for the 4229 cells (Figs 5&6).

Treating the cells with the various denaturants prior to embedding and immunolabelling, brought no significant decrease in the amount of the Au-label, suggesting once again the stability of the pCHO moiety and its presence throughout the surface of the cells (Fig 39).

(ii) Vegetative cells treated with MAbs to EA (EA2C2-1-1): B. anthracis 4229 cells were used that were grown overnight on TBAB plates. Some of these cells were boiled for 15 min, others treated with 1% SDS/ 5mM-mercaptoethanol for 30 min at 70°C. Blocks were made in LR-white and processed for EM. All samples were tested with the EA2C2-1-1 monoclonal antibody.

Gold particles were distinctly and uniformly distributed around the cells (Figs 40,41&42). Once again, this labelling was not seen intracellularly. Boiling of the cells decreased the number of gold particles, whereas cells that were treated with the urea or SDS showed a total loss of labelling (Figs 43&44), suggesting once again that there is a surface protein layer on the cells, specific for the EA-MAbs and that this layer can be removed with certain denaturants. This is in accordance with the earlier fluorescence studies.

(iii) Vegetative cells in conjunction with PA monoclonal antibody (PA1-1G7-2-1):

In our preliminary studies, there was some Au-labelling evident, but the distribution of the gold particles seemed to be rather random and scattered throughout the sections. Some labelling was routinely seen in the intercellular spaces, in the form of background nonspecific reactions. Our fluorescence studies however, gave a positive reaction and confirmed the presence of the PA moiety on the surfaces of the cells (Figs 13 thru 16). By the time we had the EM procedure well worked out, we were notified by the Army to abandon further studies on the distribution of PA and go onto EA (surface extractable antigen) related work. Therefore, no further attempts were made to resolve this lack of specific Au-labelling with PA-MAbs.

(iv) Spores + MAbs to pCHO (6G6-2-1 and 5G4-2-3):

Figs 45 thru 49 depict control, untreated spores, exposed only to Au-labelled anti-Ig. There is no deposition of Au-particles. Spores are in various stages of development, clearly showing the spore coats, cortex and inner core regions.

Spores of B. anthracis 4229, Sterne and Δ-Sterne were grown and harvested as described earlier. They were embedded and sectioned for EM studies. Immunolabelling was done directly on the grids. Very interesting patterns of gold distribution were observed. Au-particles were not present on the outer surface of the spores as seen in vegetative cells. All the activity was localized towards the central portion of the spore, an area which could be called the periphery of the germ cell, adjacent to the cortex (Figs 50 thru 53). As the spore matures and expands outwards, this activity seems to be pushed towards the periphery. The cortex layer slowly diminishes and

disappears as the spore approaches the germination stage. The distribution or presence of the Au-particles, reflective upon the presence of the pCHO layer on the core is a very logical finding , since this is the layer which eventually forms some part of the outer cell wall of the vegetative cell (Figs 54, 55 & 56).

Boiling of the spores for 30 min prior to immuno-labelling seemed to increase the amount of Au-label. The core of the boiled spore also stained more intensely with uranyl acetate and lead citrate (Figs 57 thru 63).

Figs 64 thru 67 are included not to show any immunolabelling, but simply because they were caught at fascinating times of development.

(v) Spores + MAbs to FA and PA:

Some gold particles were consistently present throughout the whole field of the sectioned spores. But this distribution was random and sparse. No conclusions could be made based on these observations (Figs 68 thru 75).

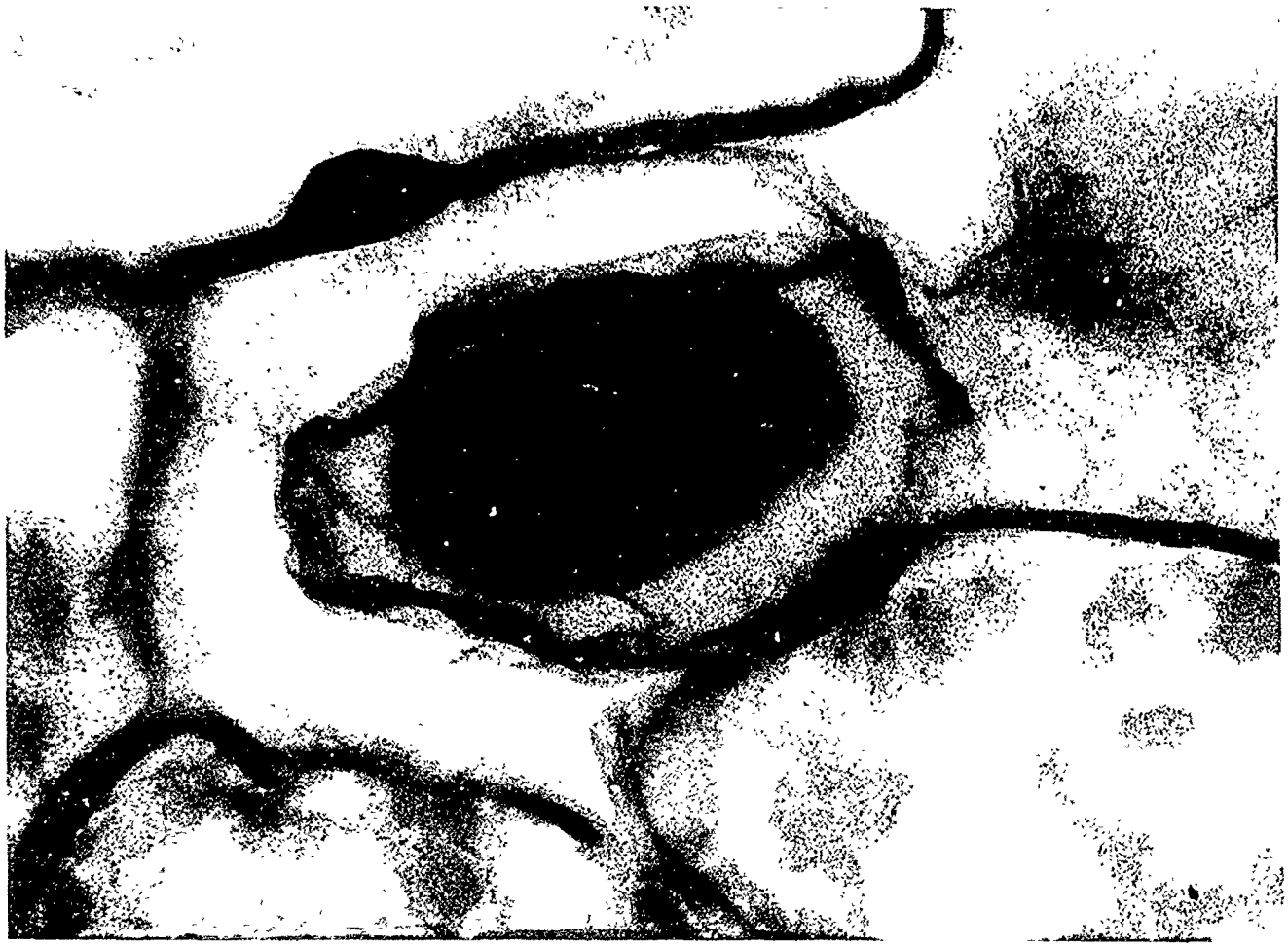


Fig. 32
B.anthracis 4229 cell showing an enclosed developing spore. No MAb was used. Only gold labelled antiIg. Mag.= 93,000

Fig.38
B.anthracis 4229 cells. This serves as a control representation. The cells were not exposed to any MAb, only to gold labelled antiIg. Note: The total lack of gold label. Mag.= 63,000

Fig. 34
B.anthracis 4229 cells treated with MAb to pCHO (5G4-2-3), followed by Au labelled antiIg. Note the extensive distribution of gold particles on the periphery of the cells, suggesting the presence of a uniform pCHO layer. Mag.= 90,000

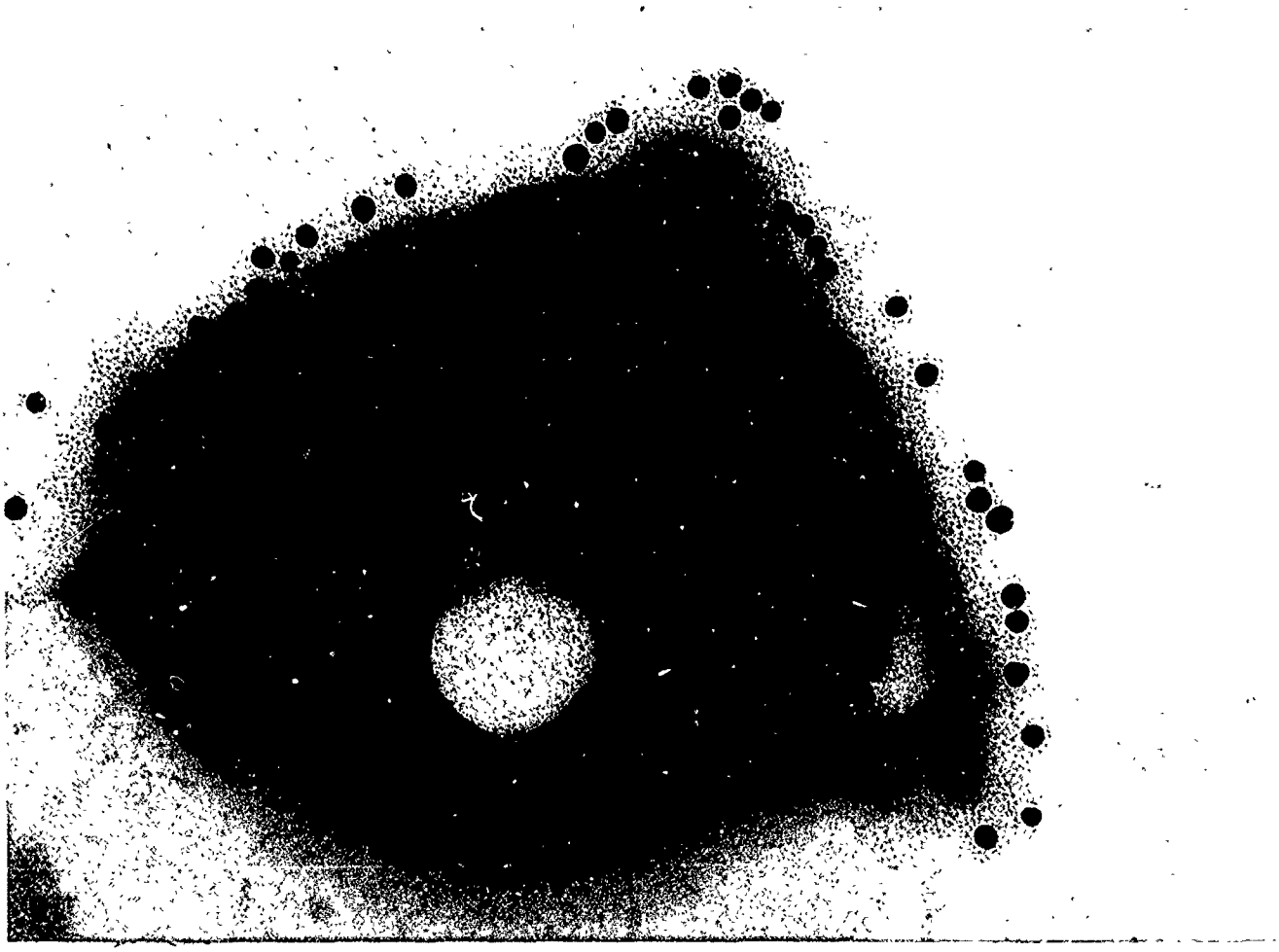


Fig. **35**
B.anthracis 4229 cells at a higher magnification showing the distribution of gold particles in response to the MAb to pCHO (564-2-3). Mag.= 150,000

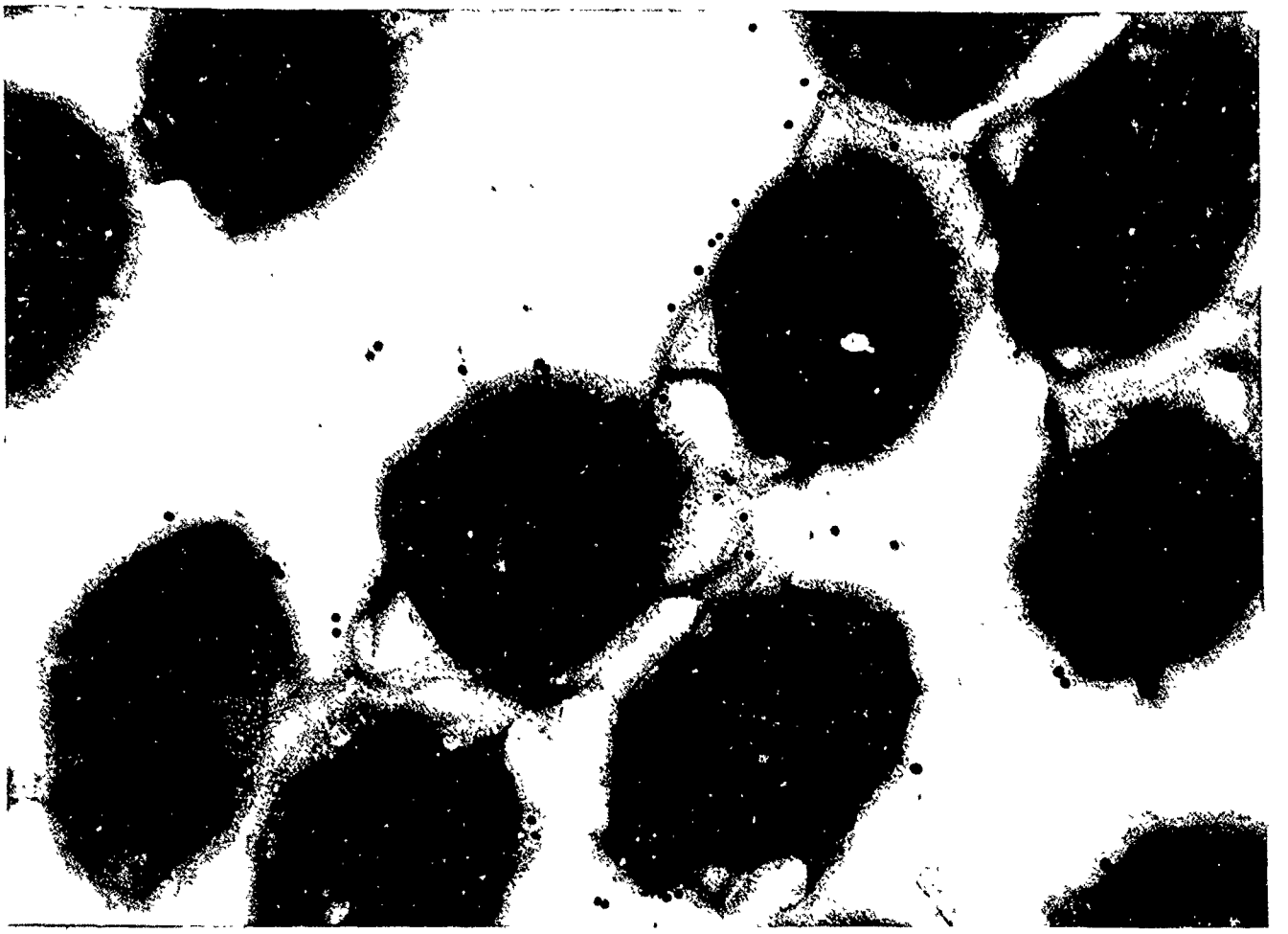


Fig. 36
B.anthracis 4229 cells treated with MAb to pCHO, 666-1-3.
Mag.= 63,000

Fig. 37
B.anthracis 4229 cells treated with MAb to pCHO, 666-1-3.
Mag.= 63,000

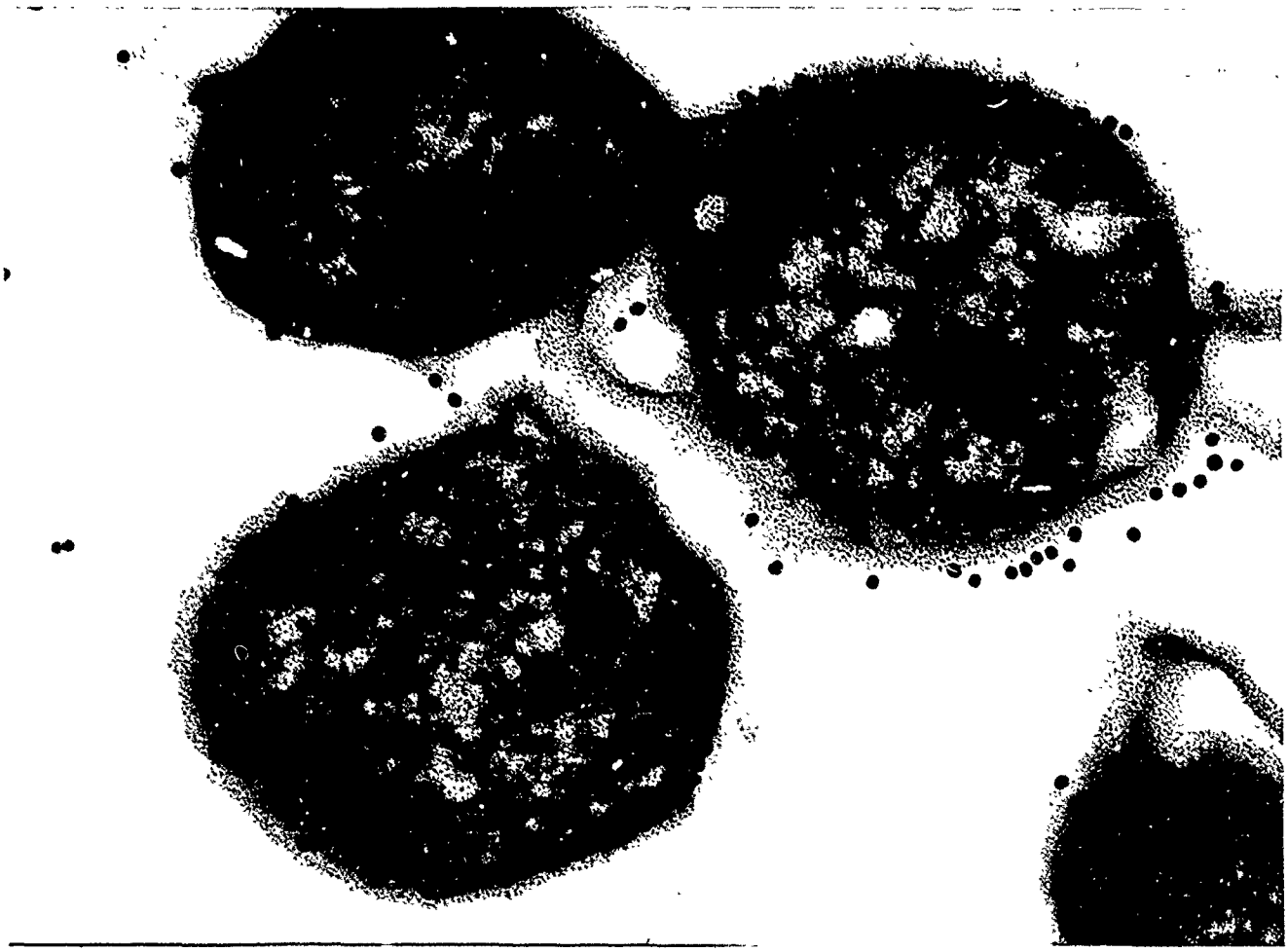


Fig. 38
B.anthracis 4229 cells, demonstrating the positive gold labelling in response to MAb against pCHO. Mag.= 96,000

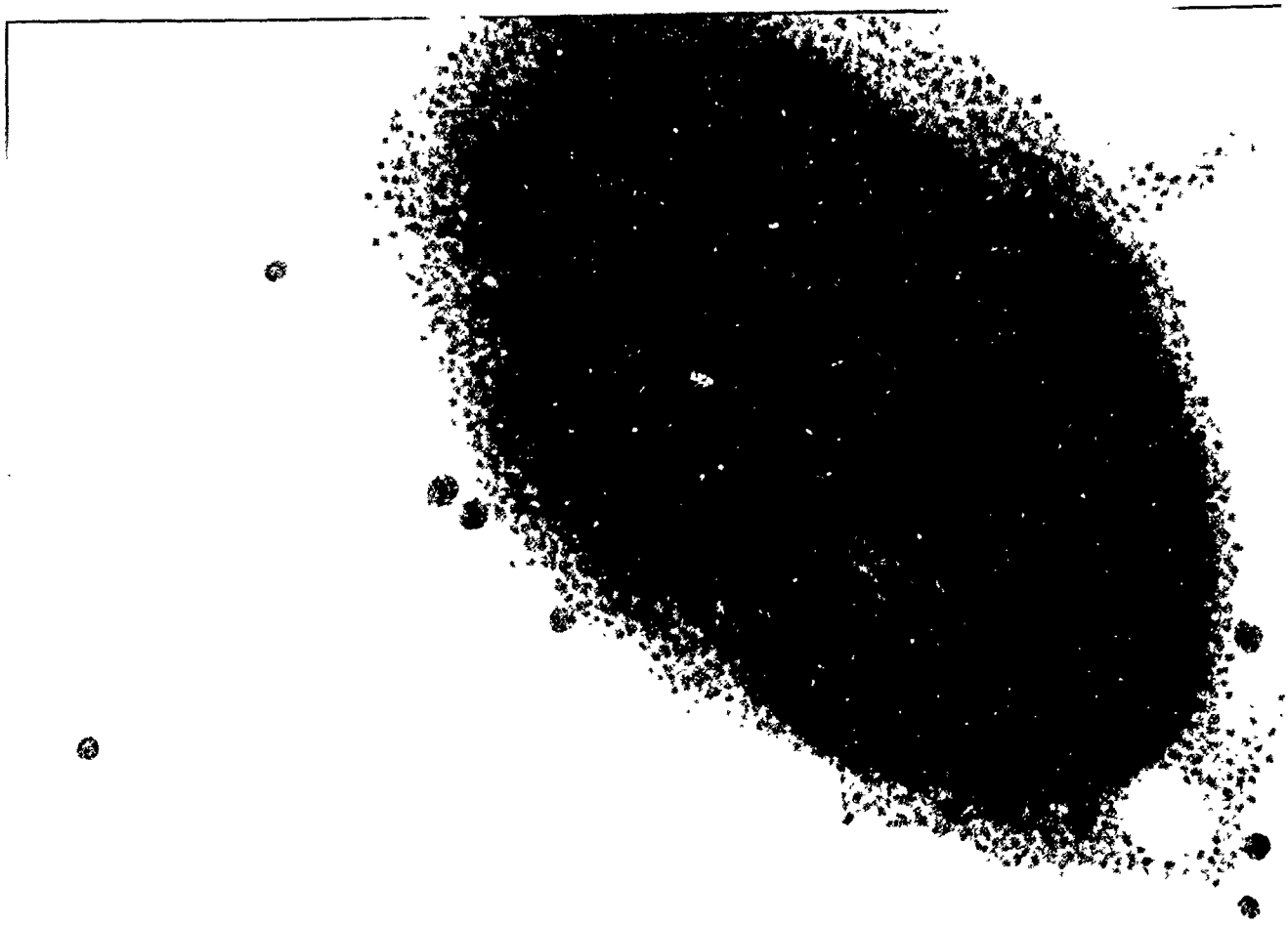


Fig.39
B.anthracis 4229 cells extracted with 1% SDS/5mM mercaptoethanol (70 ,30min) prior to treatment with SG4-1-3. Significant Au-label is still evident. Mag.= 210,000

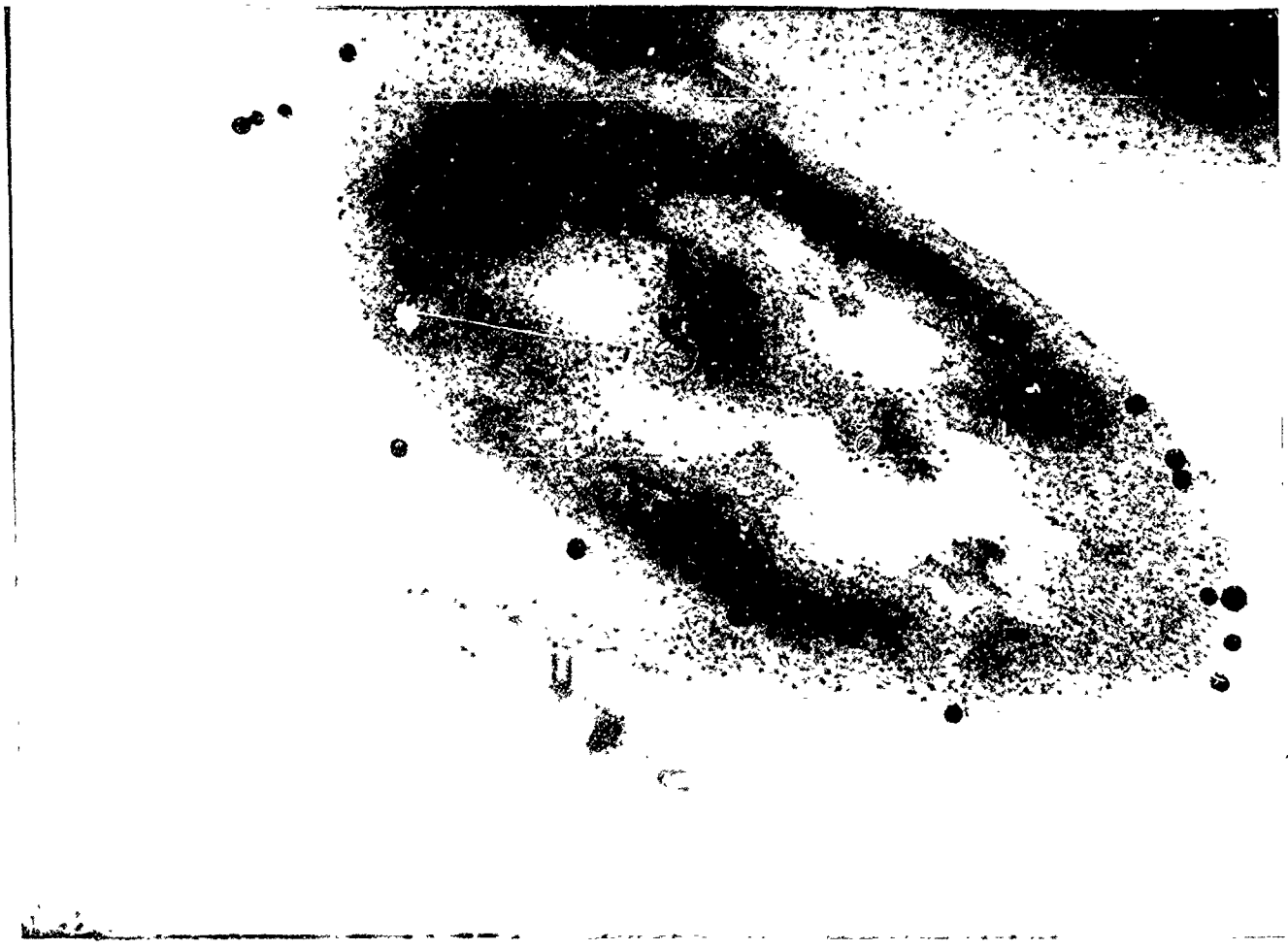


Fig. 40
B.anthracis 4229 cells treated with the MAb to EA (EA2C2-1-1), followed by Au-labelled antiIg. Note the significant, uniform surface label. Mag.= 147,000

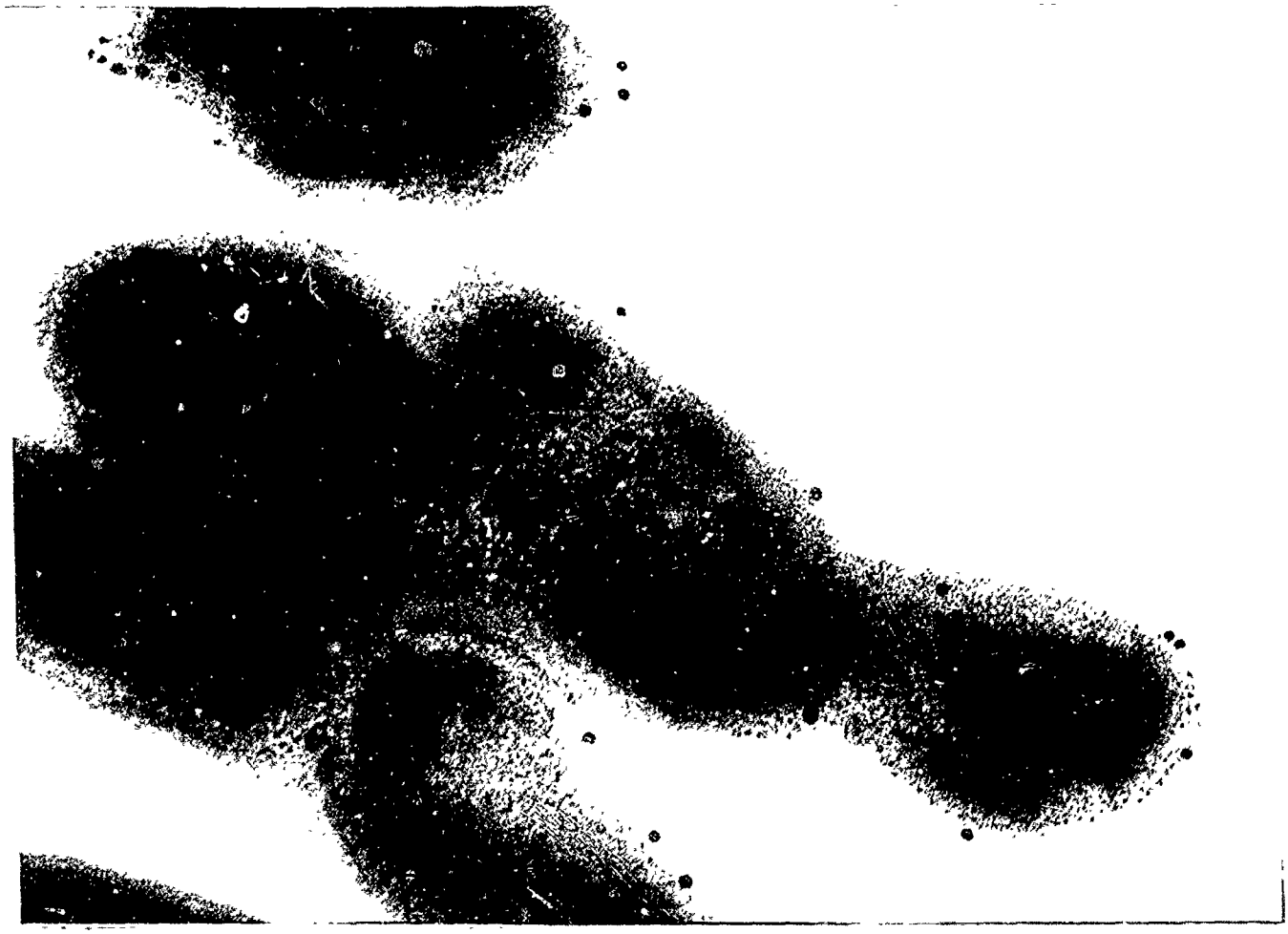


Fig. 41
B.anthracis '29 cells treated with the MAb to EA (EA2C2-1-1). An EA moiety is presence on the cell surface, hence the positive reaction with the MAb. Mag.= 94,000

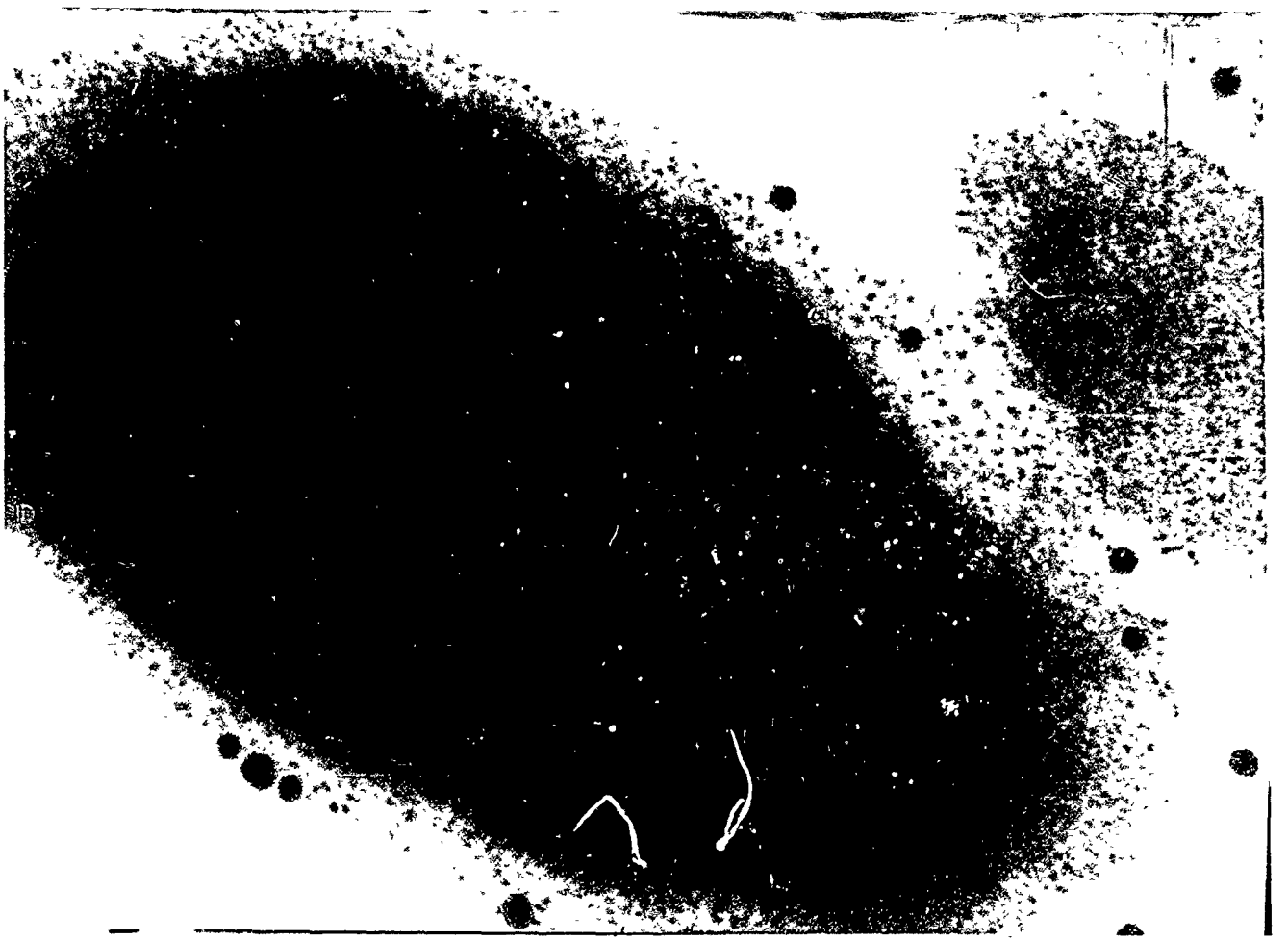


Fig. 42
B.anthracis 4229 cells in conjunction with antiEA, EA2C2-1-1.
Mag.= 212,000

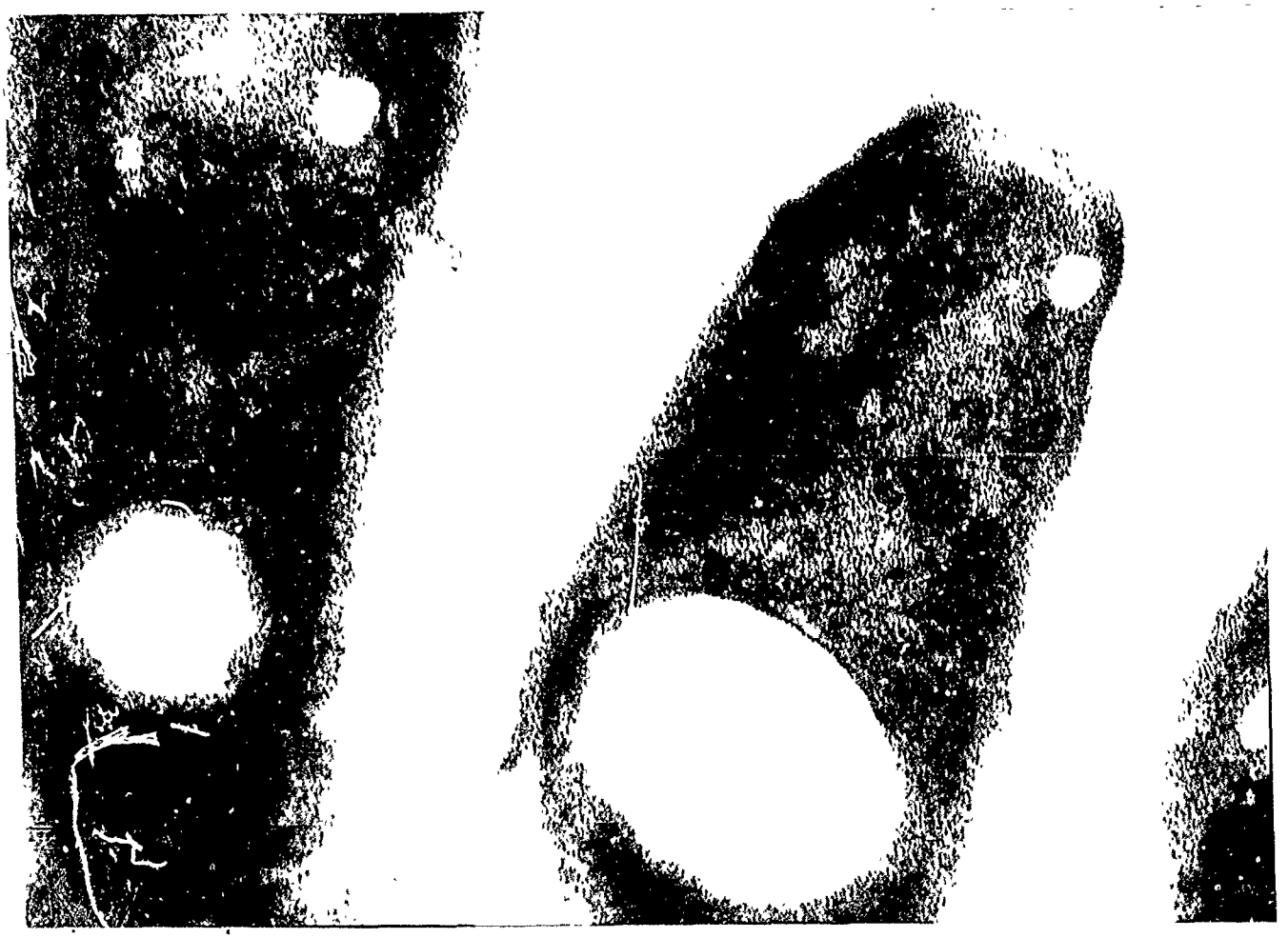
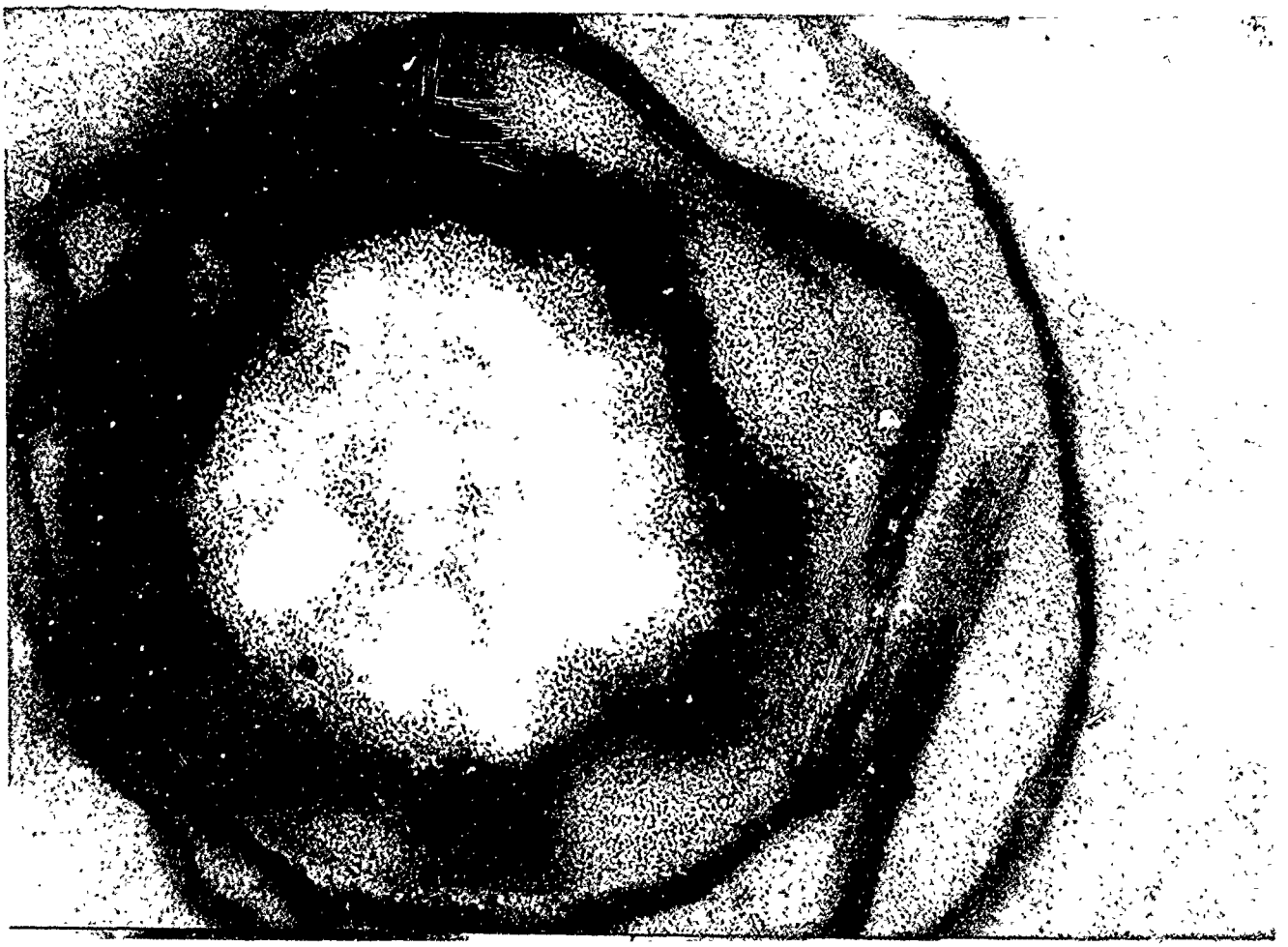


Fig. 43
B.anthracis 4229 cells extracted with 6M Urea before processing for the EM studies. It appears that the surface antigen is lost by this treatment and hence there is no labelling when exposed to the MAB to EA. Mag.= 96,000

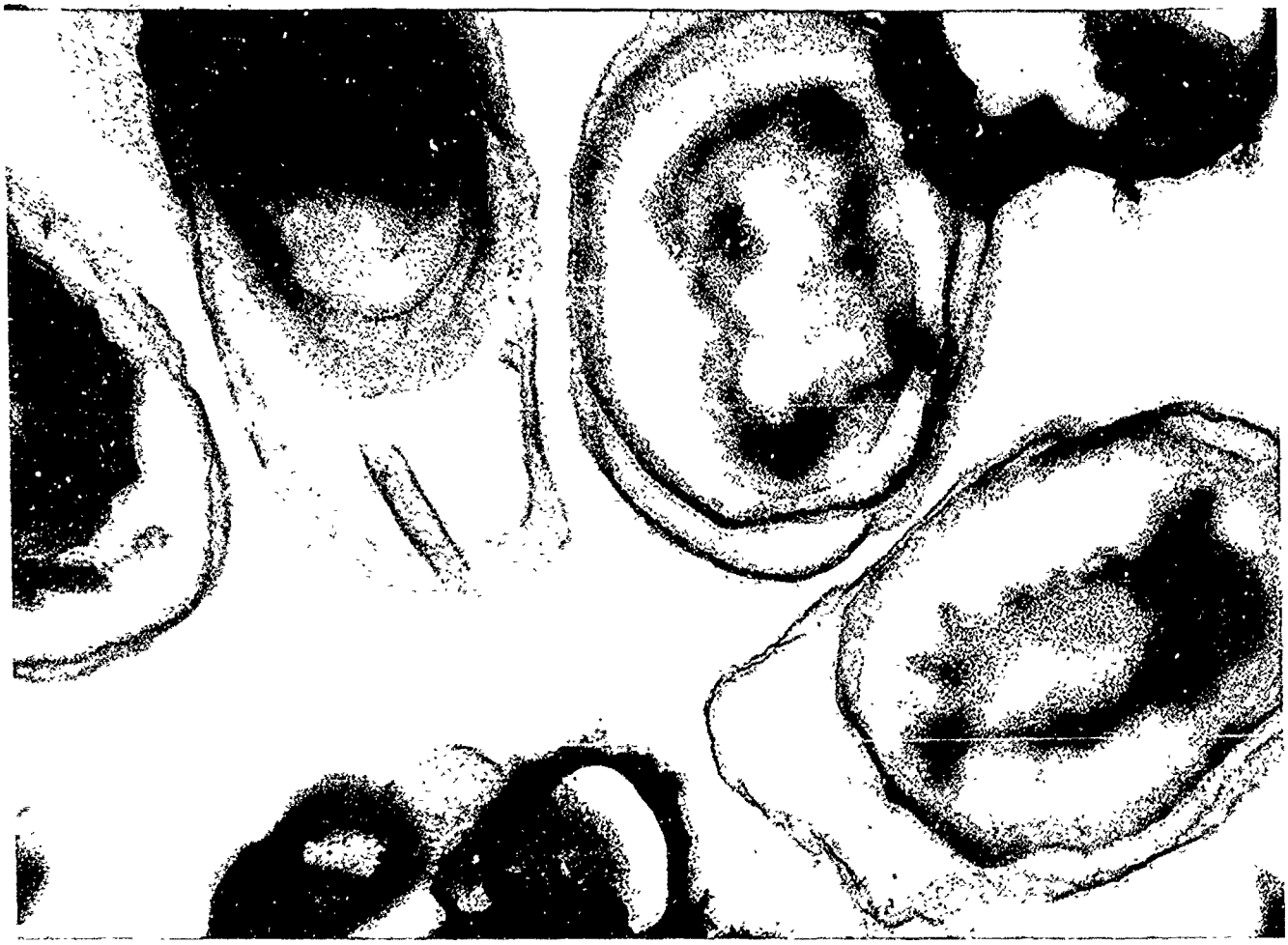
Fig. 44
B.anthracis 4229 cells extracted with 1% SDS/5mM mercaptoethanol and treated with MAbs to EA. The treatment seems harsh for this particular antigen. There is total absence of labelling when tested with EA2C2-1-1. Mag.= 90,000

Fig. 45



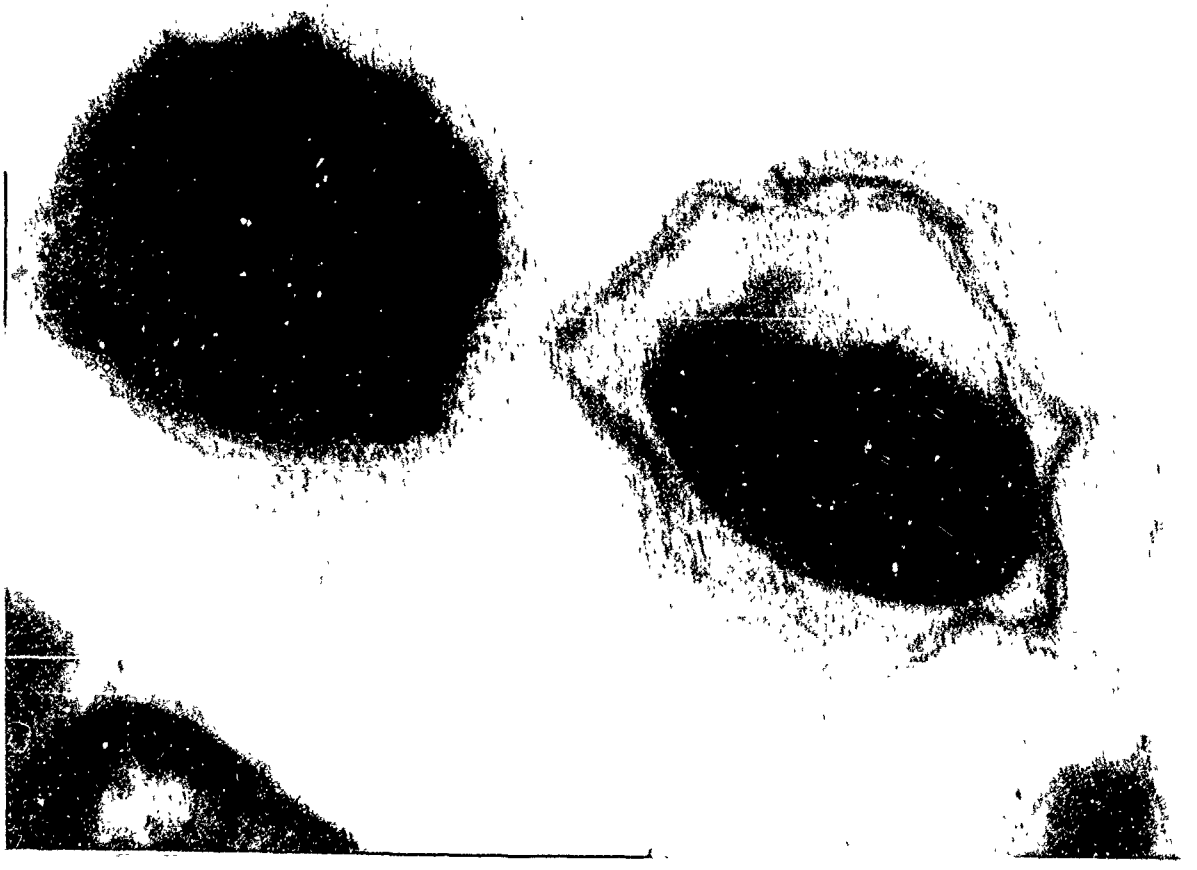
B.anthracis Sterne spore. Control. No exposure to any MAbs, only Au-labelled antiIg. Mag.=149,000

Fig. 46



B.anchracis Sterne spores. Control. No immunolabelling.
Mag.= 63,000

Fig. 47

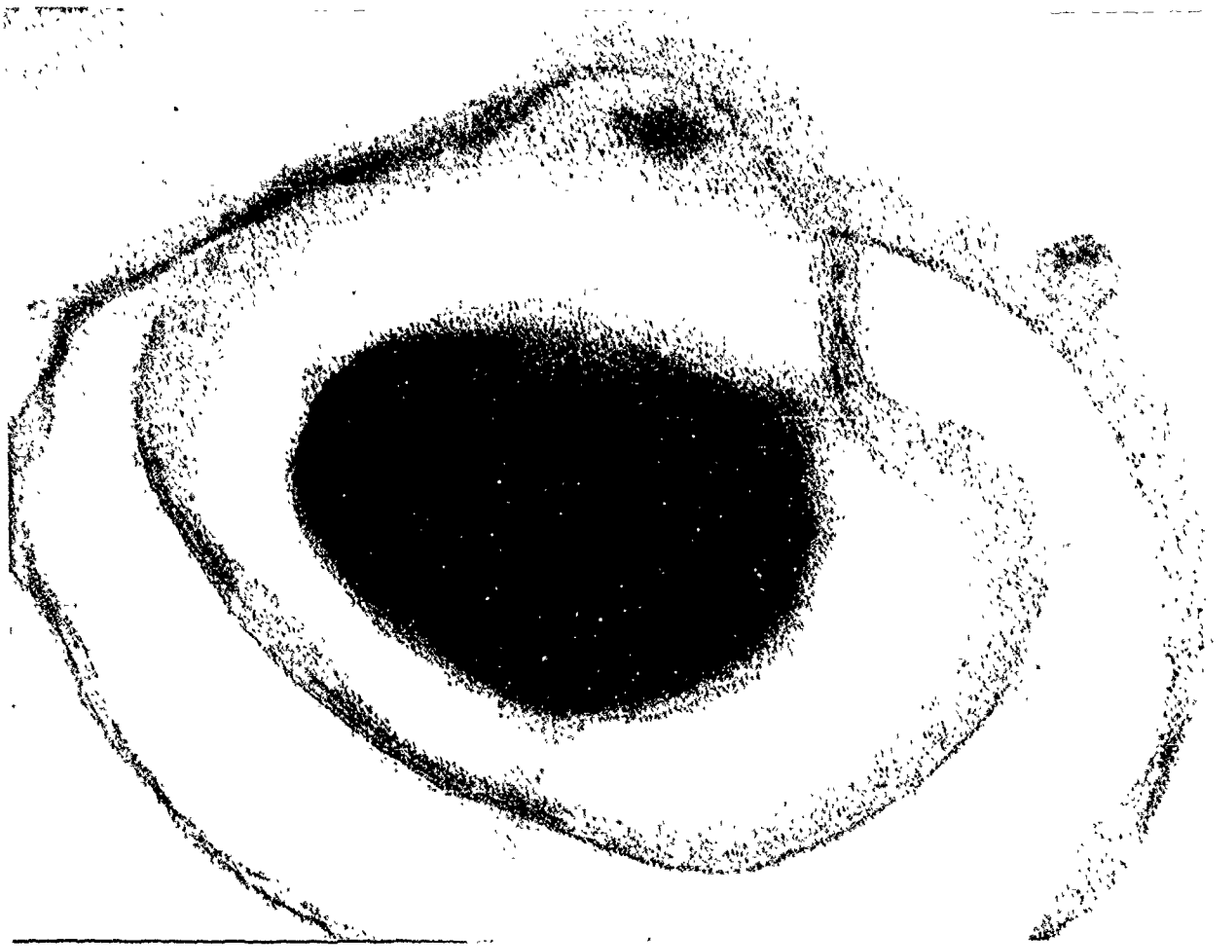


B.anthracis 4229 control, unboiled spores. Mag.= 95,000

Fig. 48

Δ-Sterne spores. Large and well developed. The cortex layer is diminished and seems to be merging with the spore-coats. Control, not treated with any MAbs. Mag.= 63,000

Fig.49



B.anthracis Δ-Sterne spore. A control specimen, not treated with any immunoglobulins. Mag.= 149,000

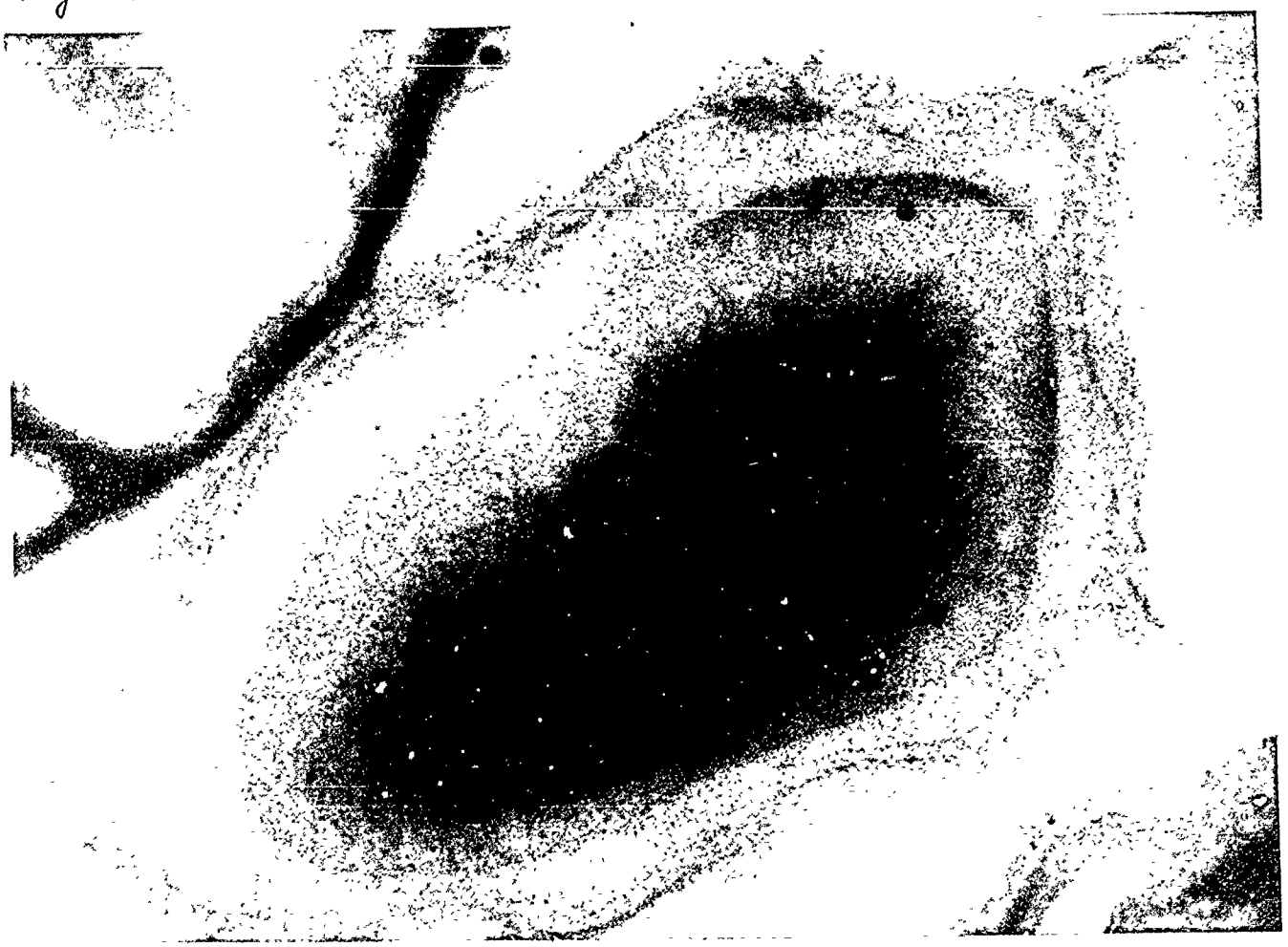
Fig. 50

B.anthracis Sterne spores treated with anti-pCHO (666-1-2).
Mag.= 65,000

Fig 51

B.anthracis 4229 cell and spore in the same field. Exposed to 5G4--2-3, an anti-pCHO. Mag.= 95,000

Fig. 52

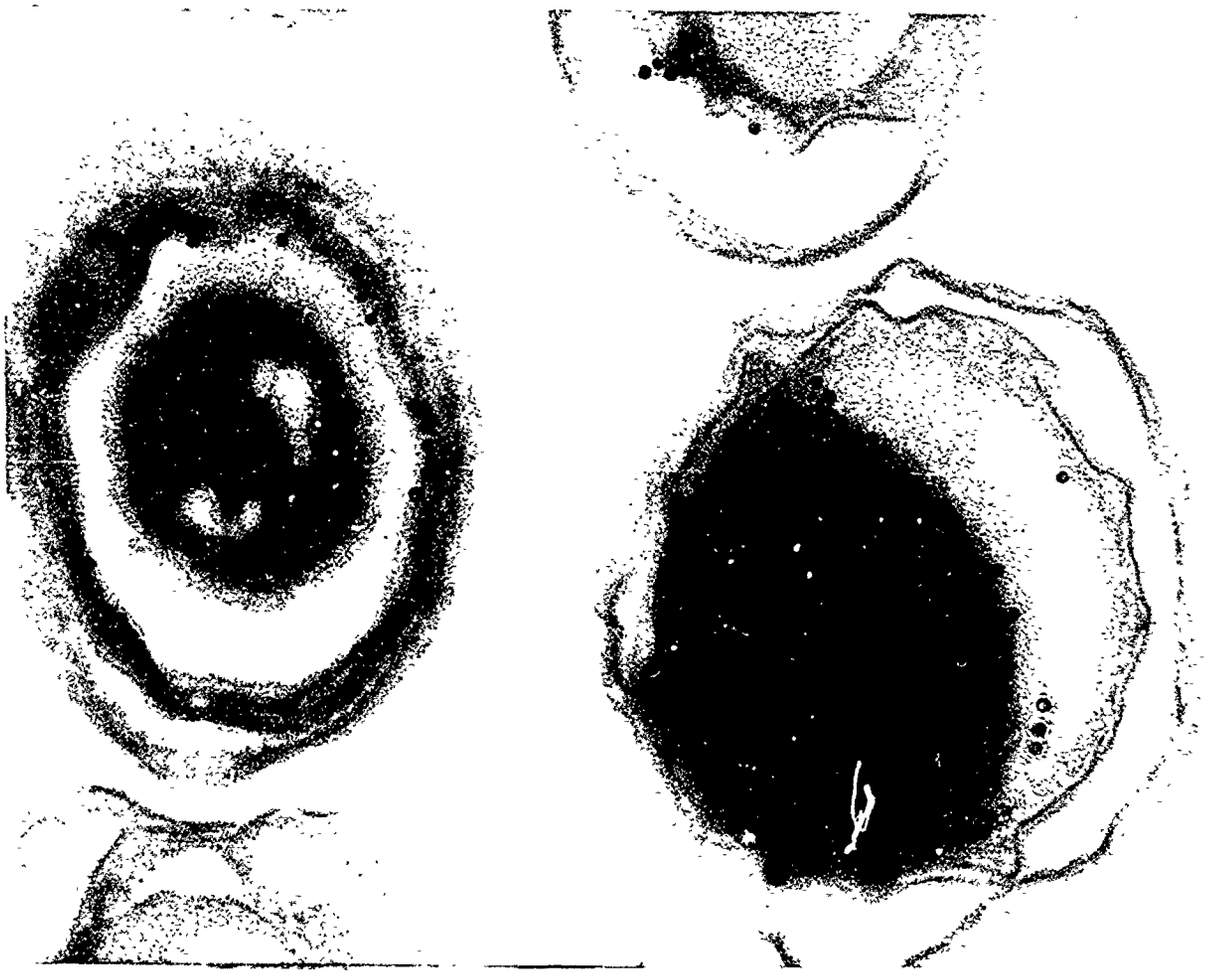


B.anthracis 4229 spore, unboiled and treated with 666-2-1.
Mag.= 147,000

Fig. 53

B.anthracis Sterne, unboiled spore, treated with 66-1-2 anti-pCHO
As the spore transforms into a vegetative cell, the labelling is
seen on the periphery of the cell. Mag.= 148,000

Fig. 54

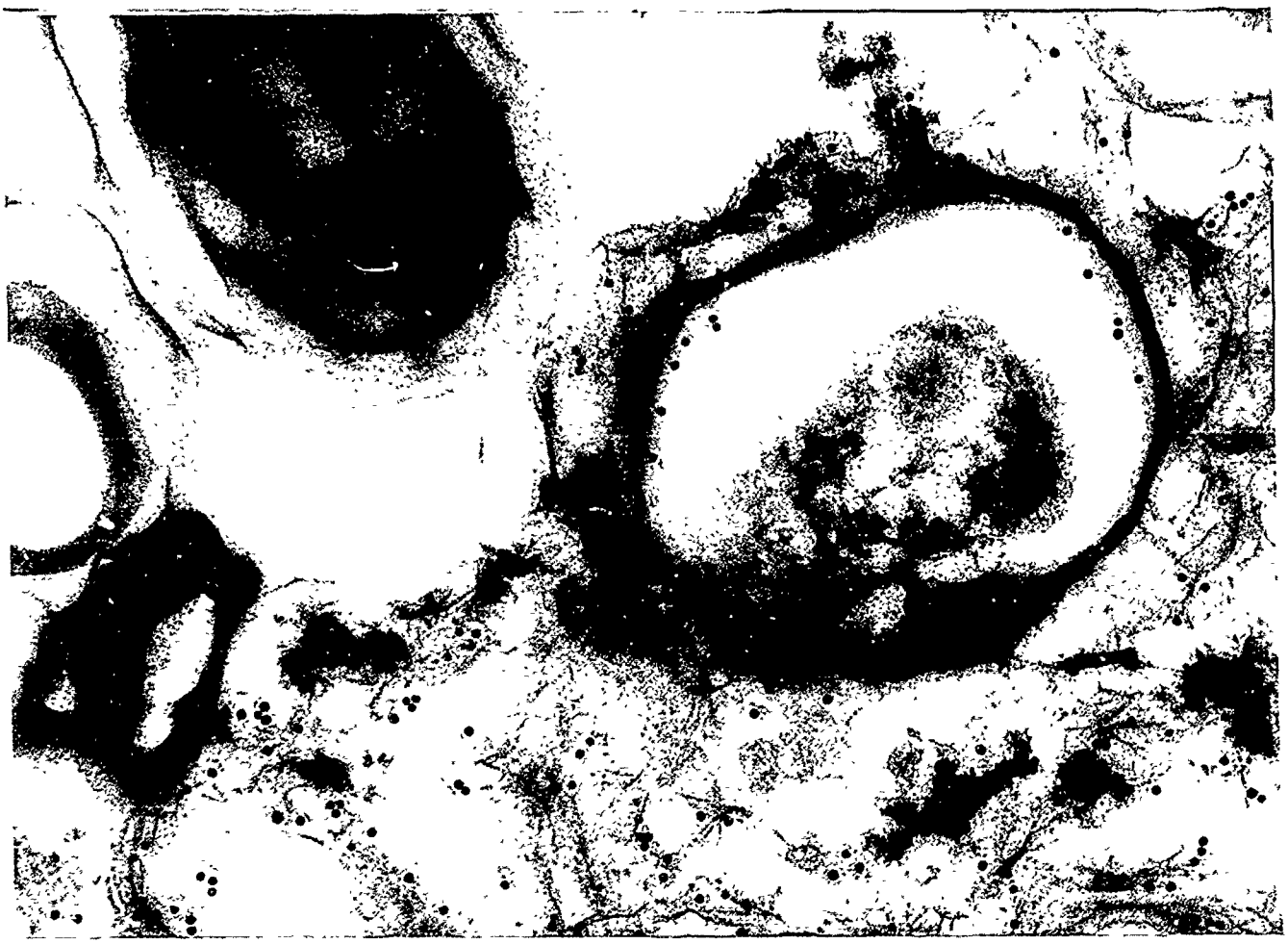


B.anthracis Sterne spores boiled for 15 minutes, before exposing to 564-2-3 anti-pCHO. Note the labelling next to the core of the spore. This activity moves outwards as the spore develops.
Mag.= 96,000

Fig. 55

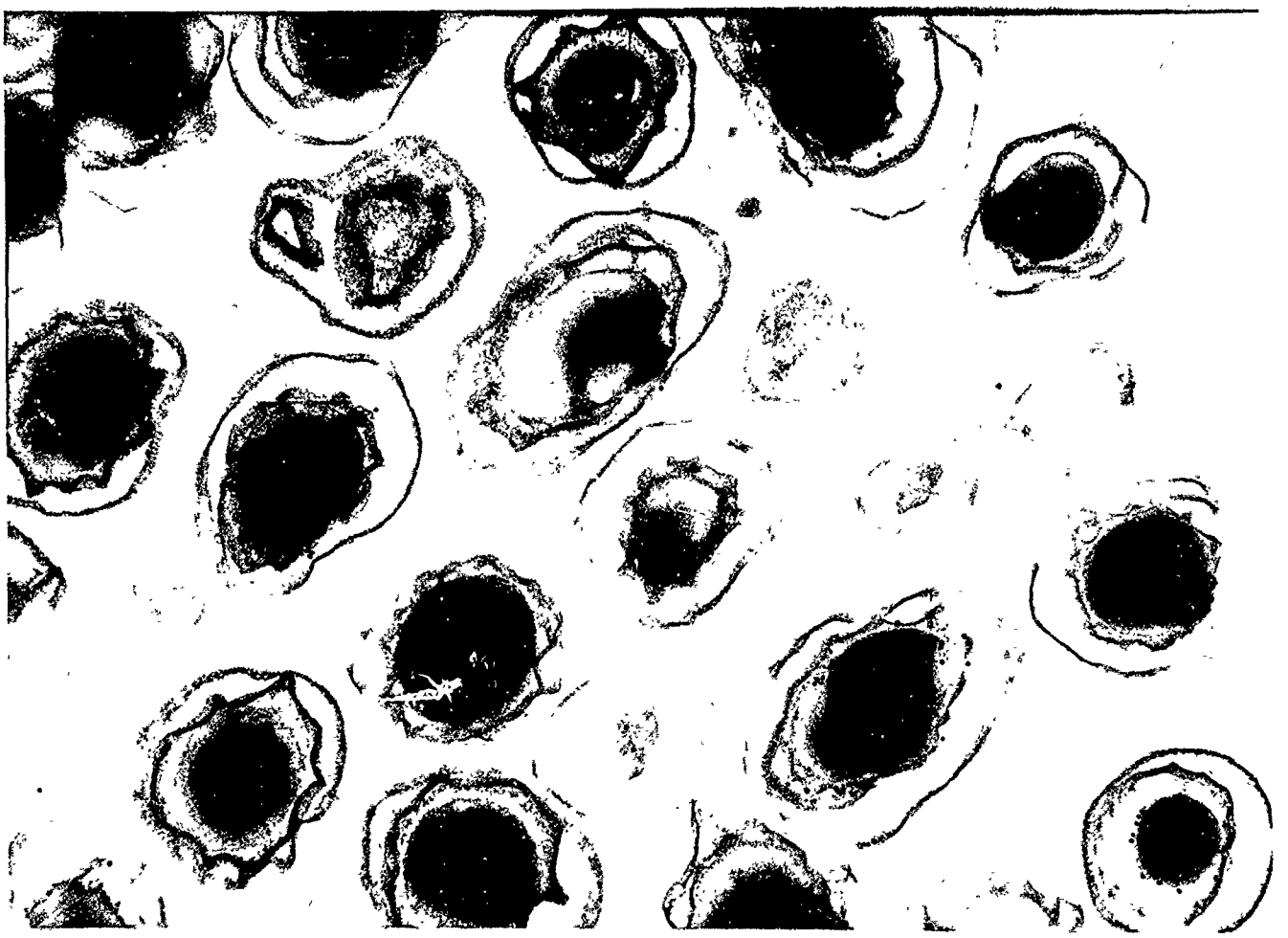
Fig.
B.anthracis 4229 spore boiled for 15 min then subjected to 5G4-2-3 MAb to pCHO. The spore (bottom of the micrograph) has gold particles around the core, whereas the disrupted cell displays labelling on the cell wall. This is a truely appropriate finding, because the core of the spore ultimately grows out and becomes the outermost region of the cell. Mag.= 102,000

Fig. 56



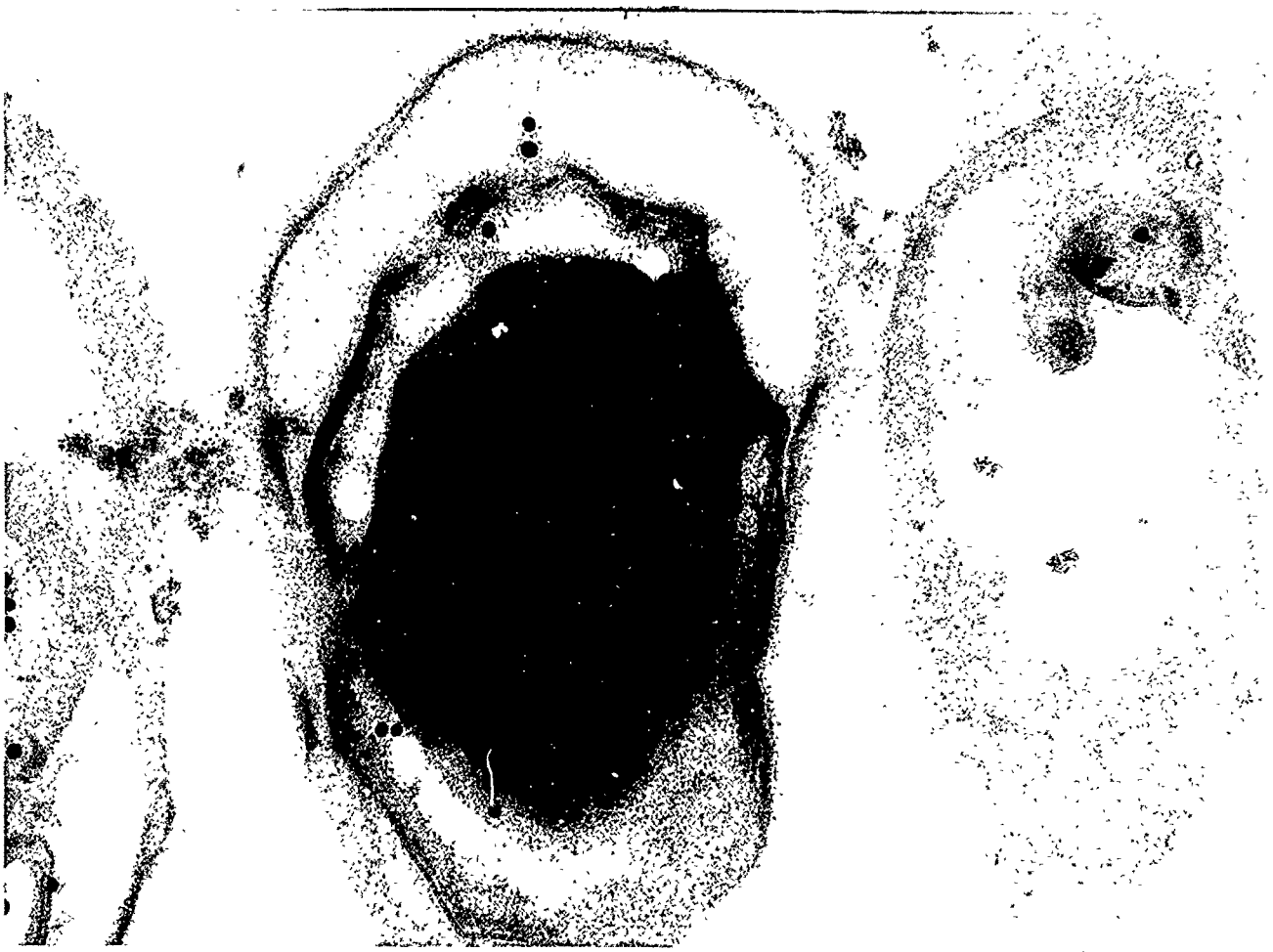
A field showing a spore, adjacent to a cell. Note the lack of gold distribution on the spore. The gold particles, indicative of a pCHO layer are quite prominent on the periphery of the cell as well as the cell wall remnants in the backgrourd. Mag.= 64,000

Fig. 57



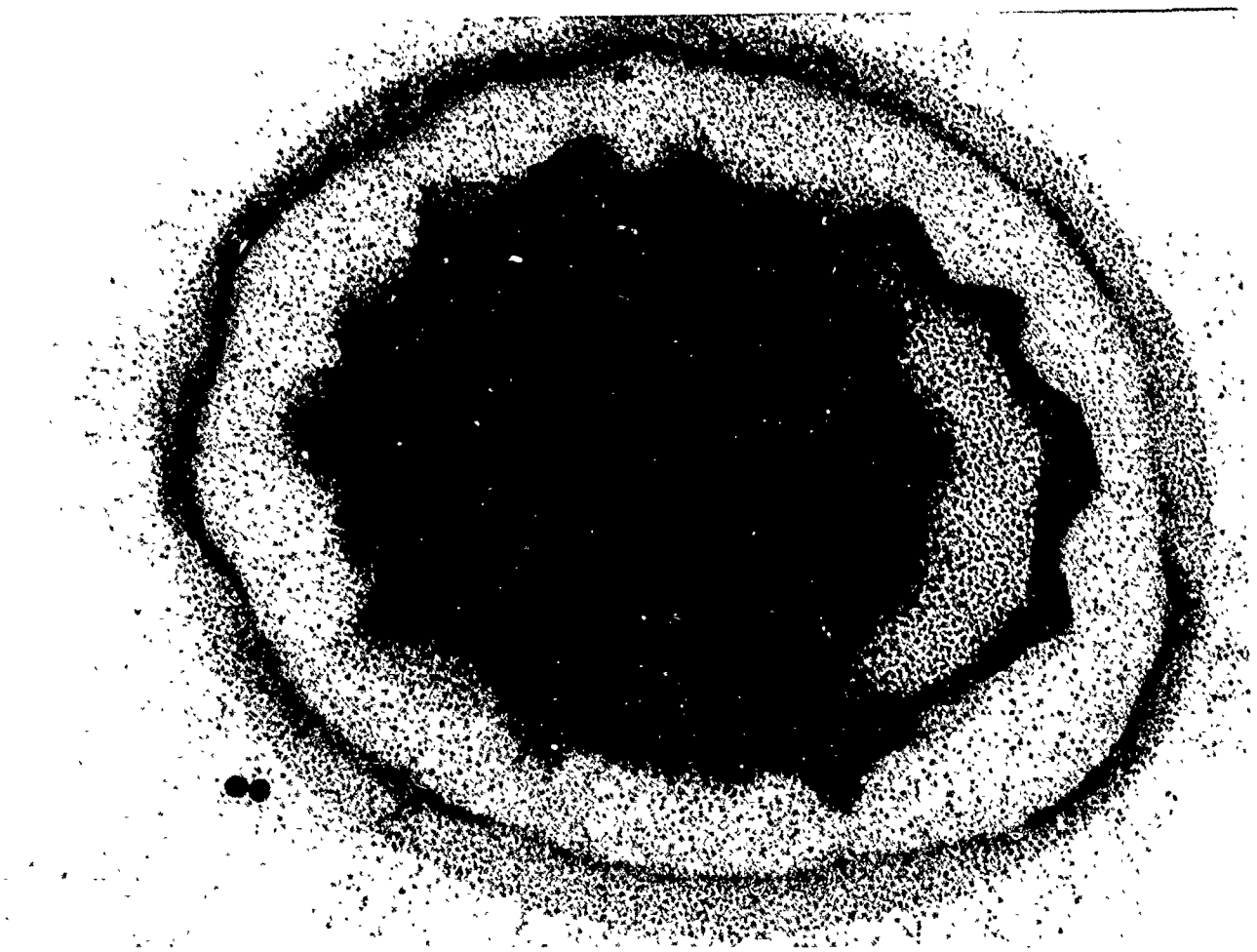
B.anthracis Sterne spores, boiled and exposed to 6G6-1-2 anti-pCHO. Spores in several different stages of developement are shown. Mag.= 32,000

Fig. 58



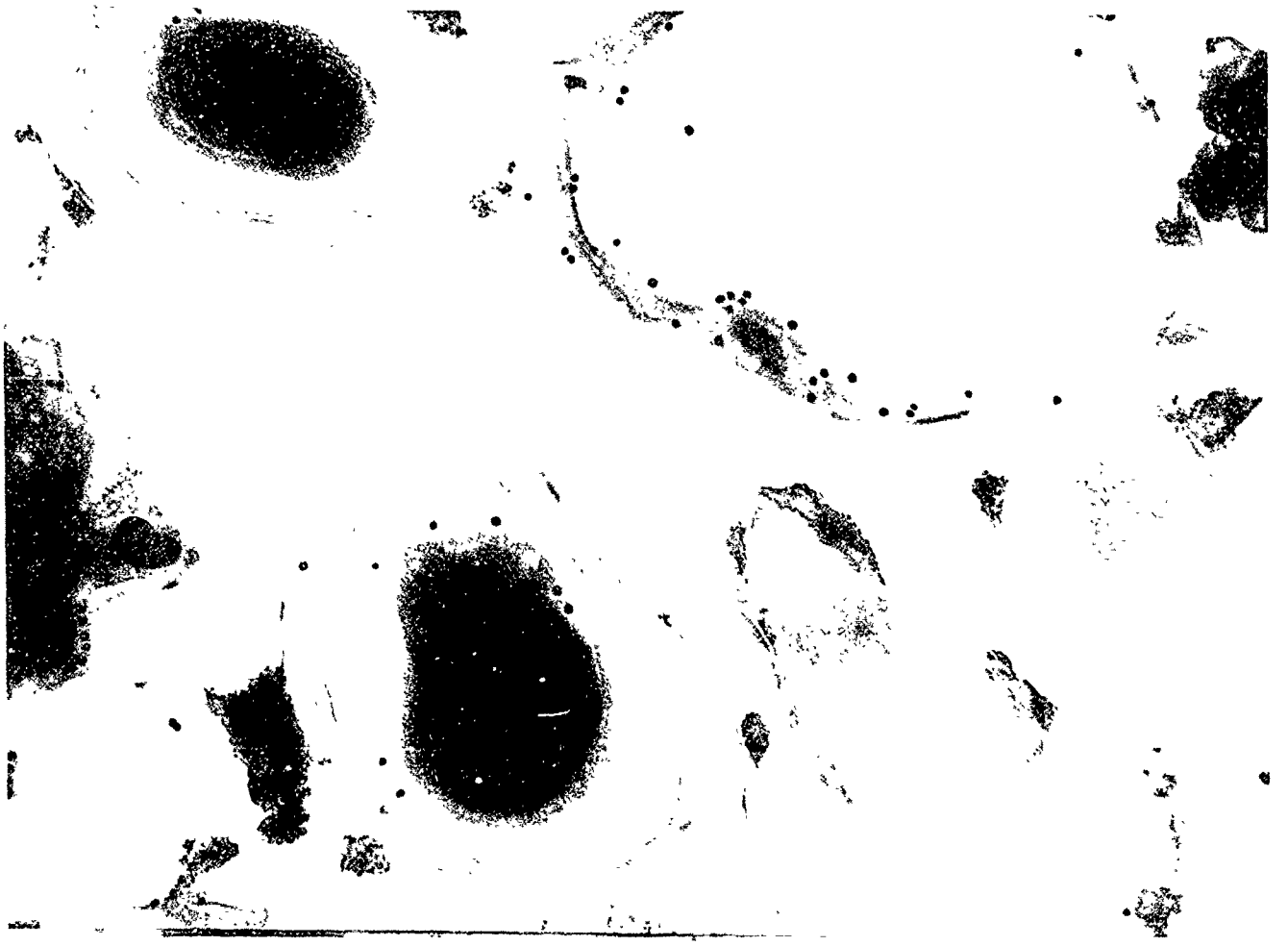
B.anthracis Sterne spores boiled for 15 minutes and treated with 6G6-1-2 anti-pCHO. Note the presence of Au particles around the core, in close proximity of the cortex. Mag.= 98,000

Fig. 59



B.anthracis Sterne spore, boiled before exposing to anti-pCHO (666-1-2). Mag.= 150,000

Fig. 60



B.anthracis Δ-Sterne spores, boiled and treated with 6G6-2-1 anti-pCHO. Note: The deposition of Au-particles on the periphery of the core. Mag.= 95,000

Fig. 61

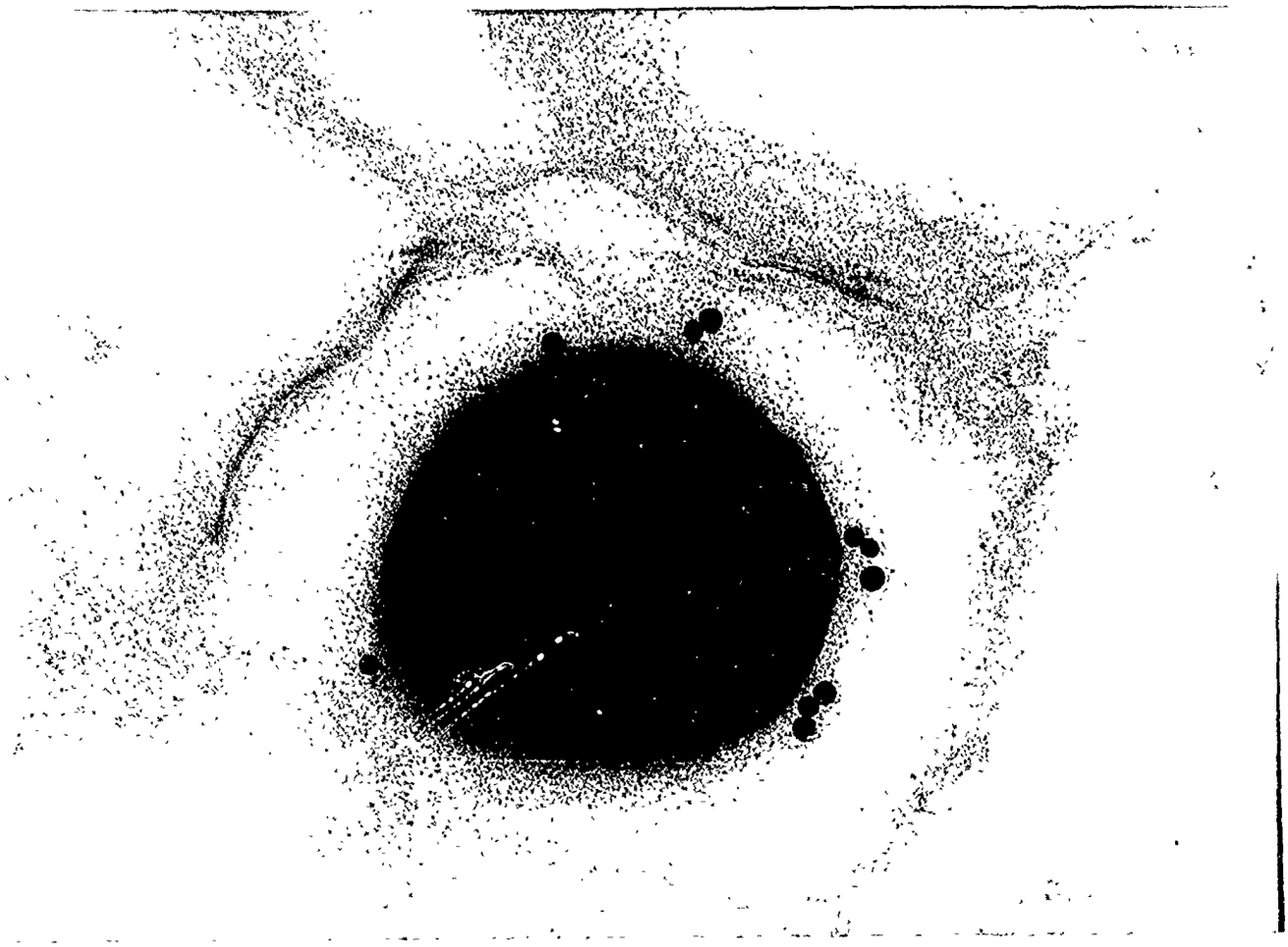
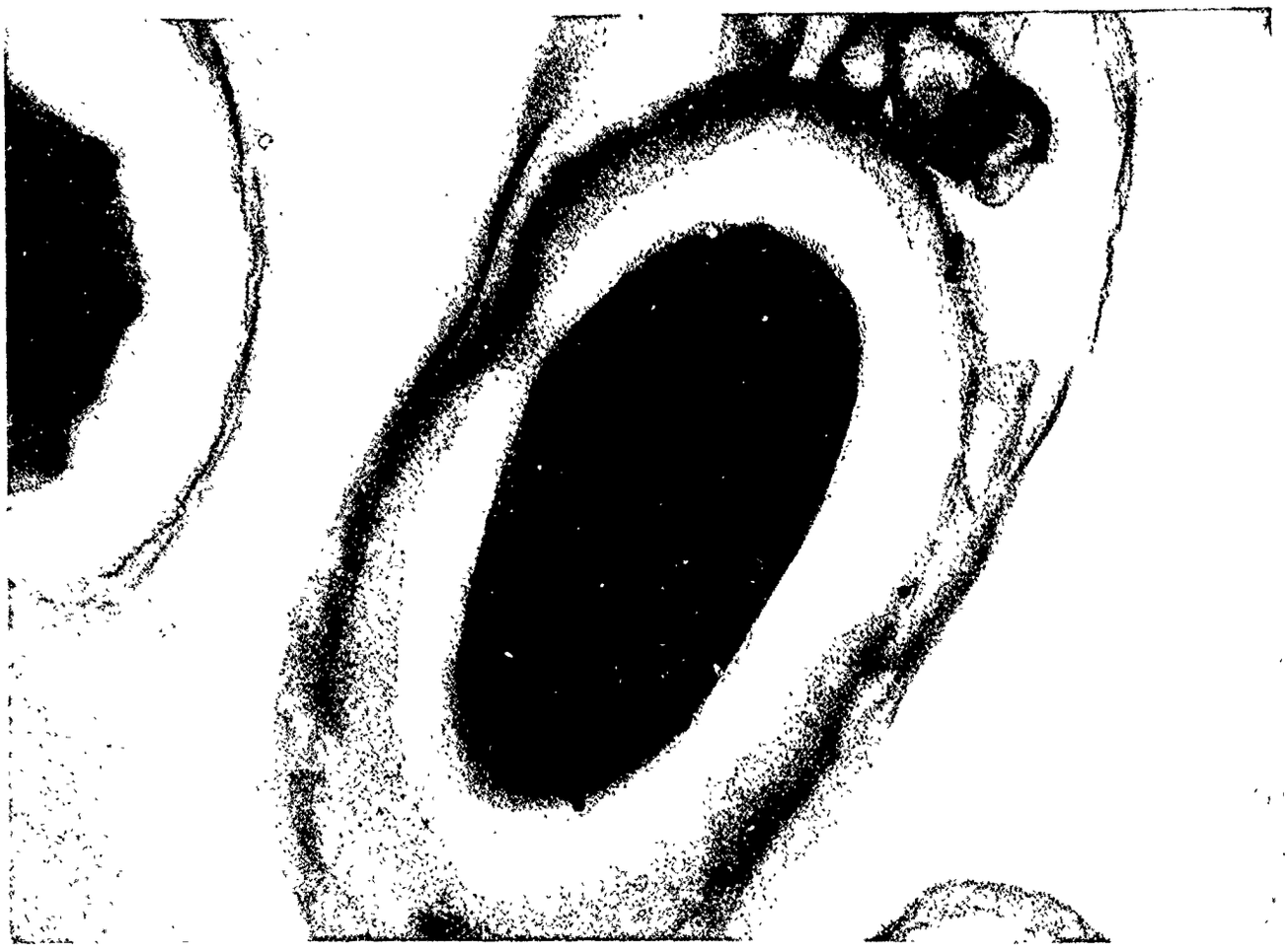


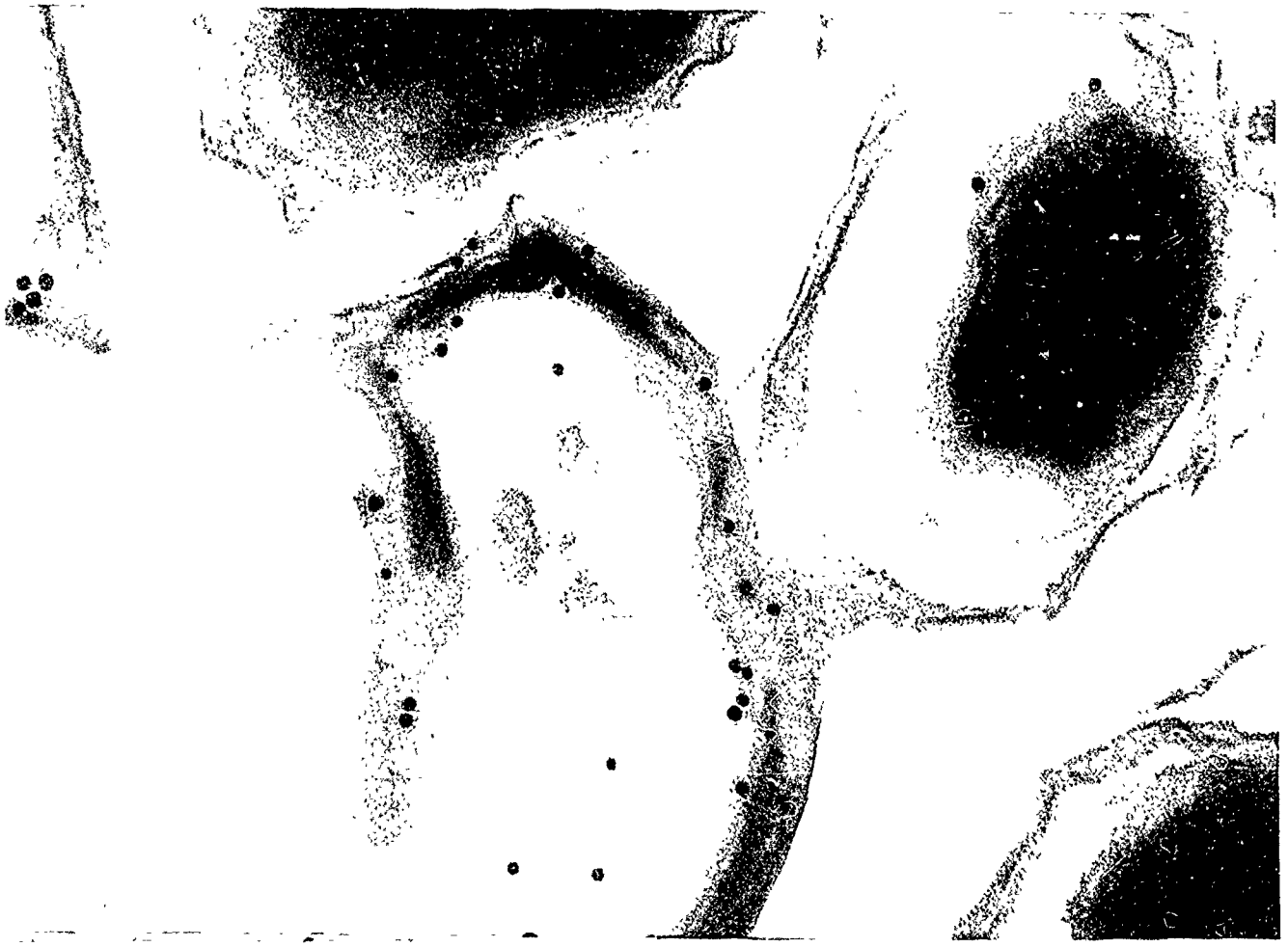
Fig.
B.anthracis 4229 boiled spore showing the Au-particles in the vicinity of the central core. 6G6-2-1 antipCHO was employed.
Mag.= 146,000

Fig. 62



B.anthracis Sterne spores boiled for 15 minutes and treated with MAb to pCHO (564-2-3). Note: Significant Au-labelling around the core of the spore. An area, destined to become the outer cell wall of the vegetative cell. Mag.= 132,000

Fig. 63



B. anthracis Δ-Sterne spore, boiled and treated with 584-2-3. The core seems to stain darker when boiled. Mag.= 96,000

Fig 64

Fig.
B.anthracis 4229 control cell shown initiating its process of division, leading to a forespore. No MAb treatment. Mag.= 96,000

Fig. 65

B.anthracis 4229 control cell, undergoing division. Mag.= 147,000

Fig.66

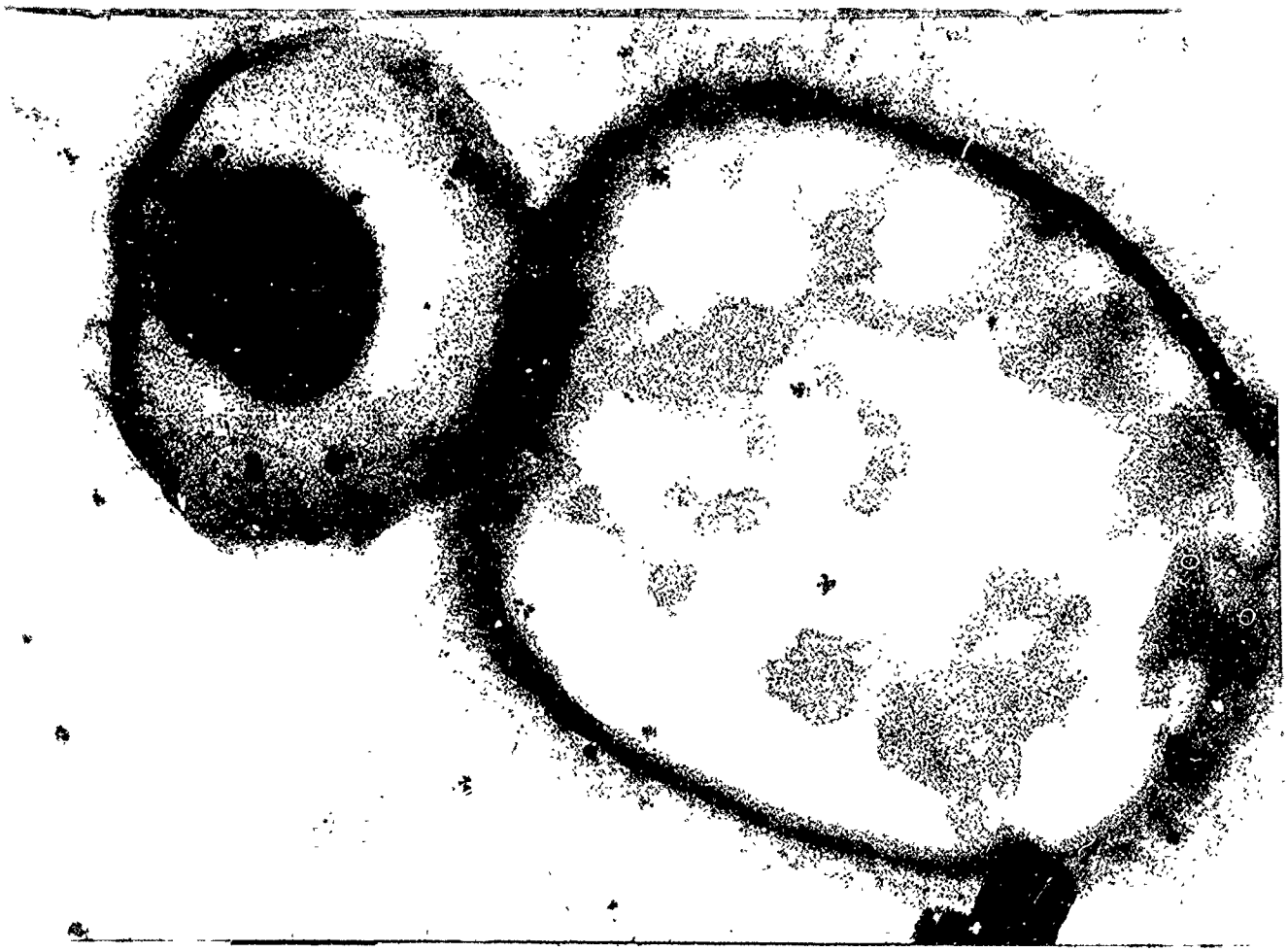
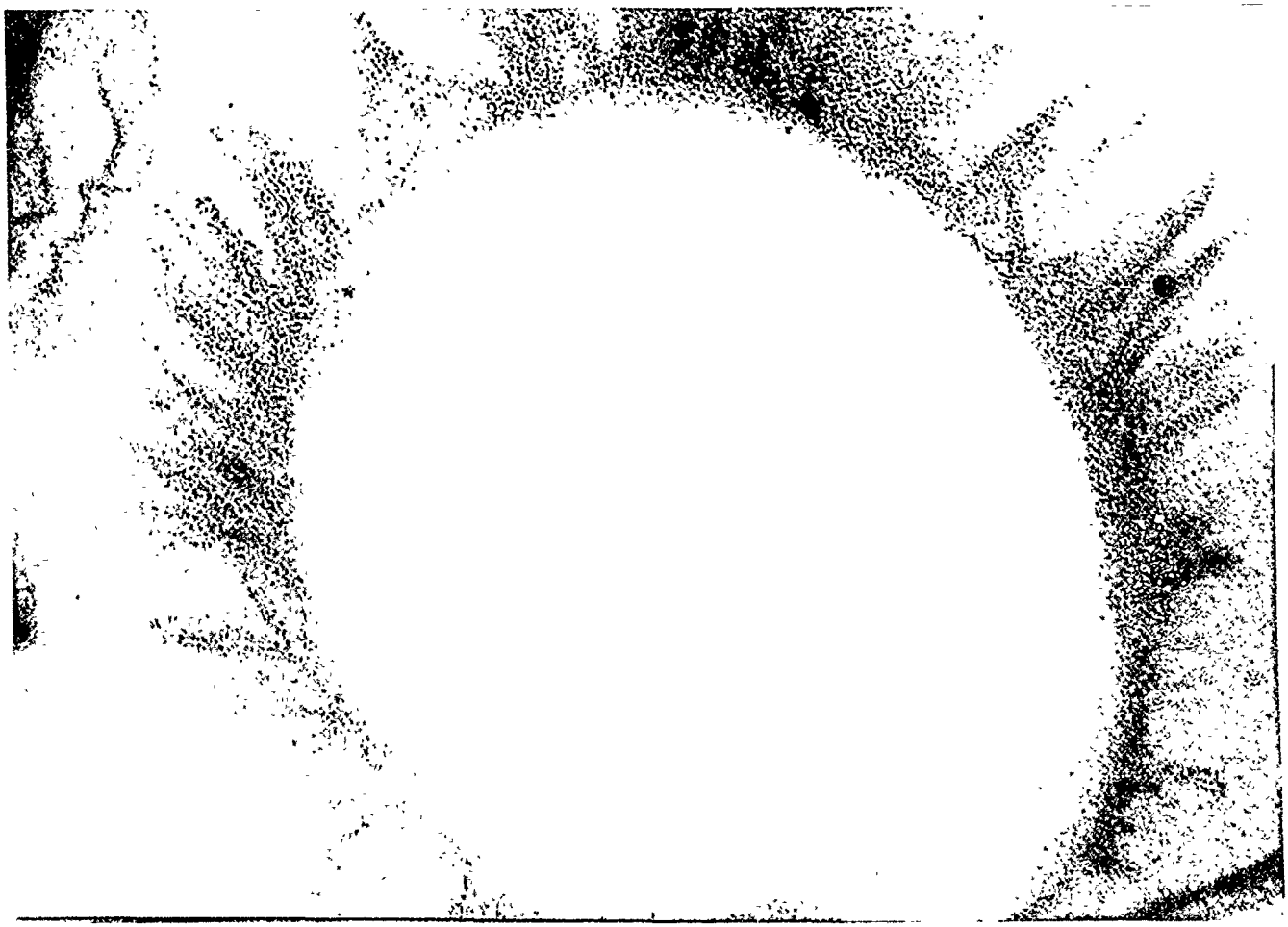


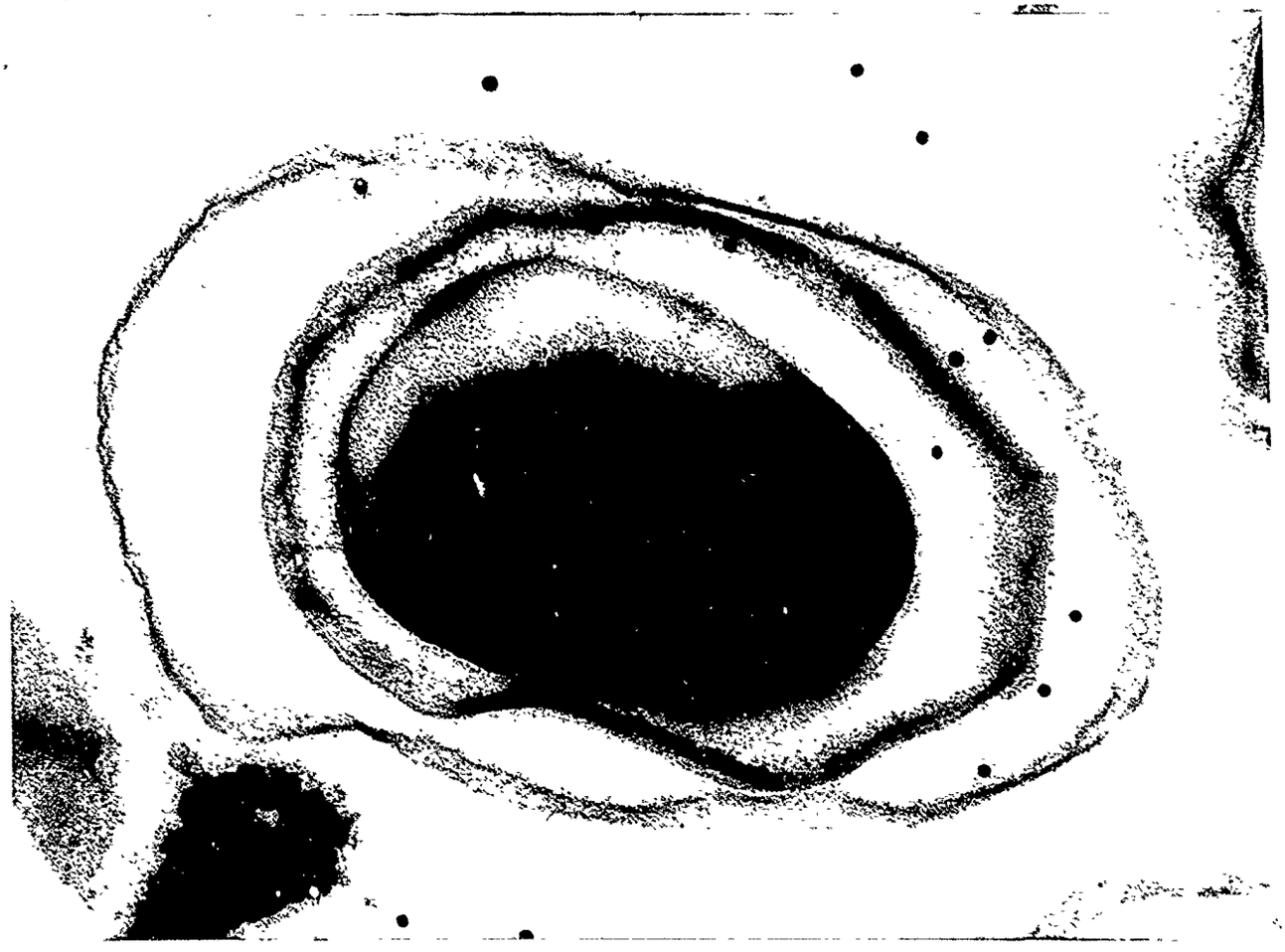
Fig.
A section of B.anthracis 4229 showing the formation of a forespore. The smaller sphere represents a developing spore. Note gold labelling around the inner core of the spore in contrast to the labelling present on the vegetative cell. 5G4-2-3 antipCHO was employed. Mag.= 93,000

Fig. 67



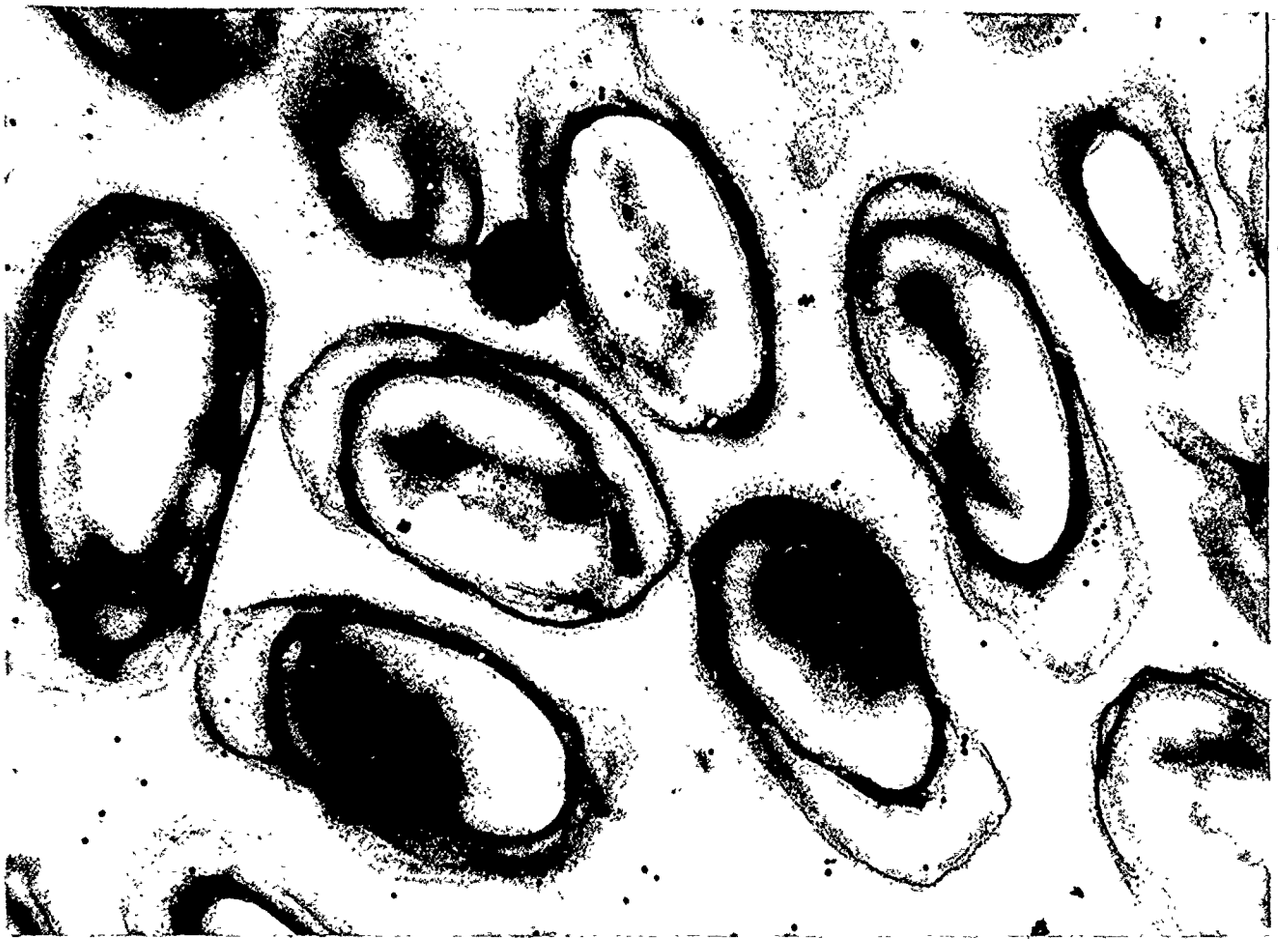
B. anthracis Δ-Sterne spore displaying a unique appearance.
Mag.= 147,000

Fig. 68



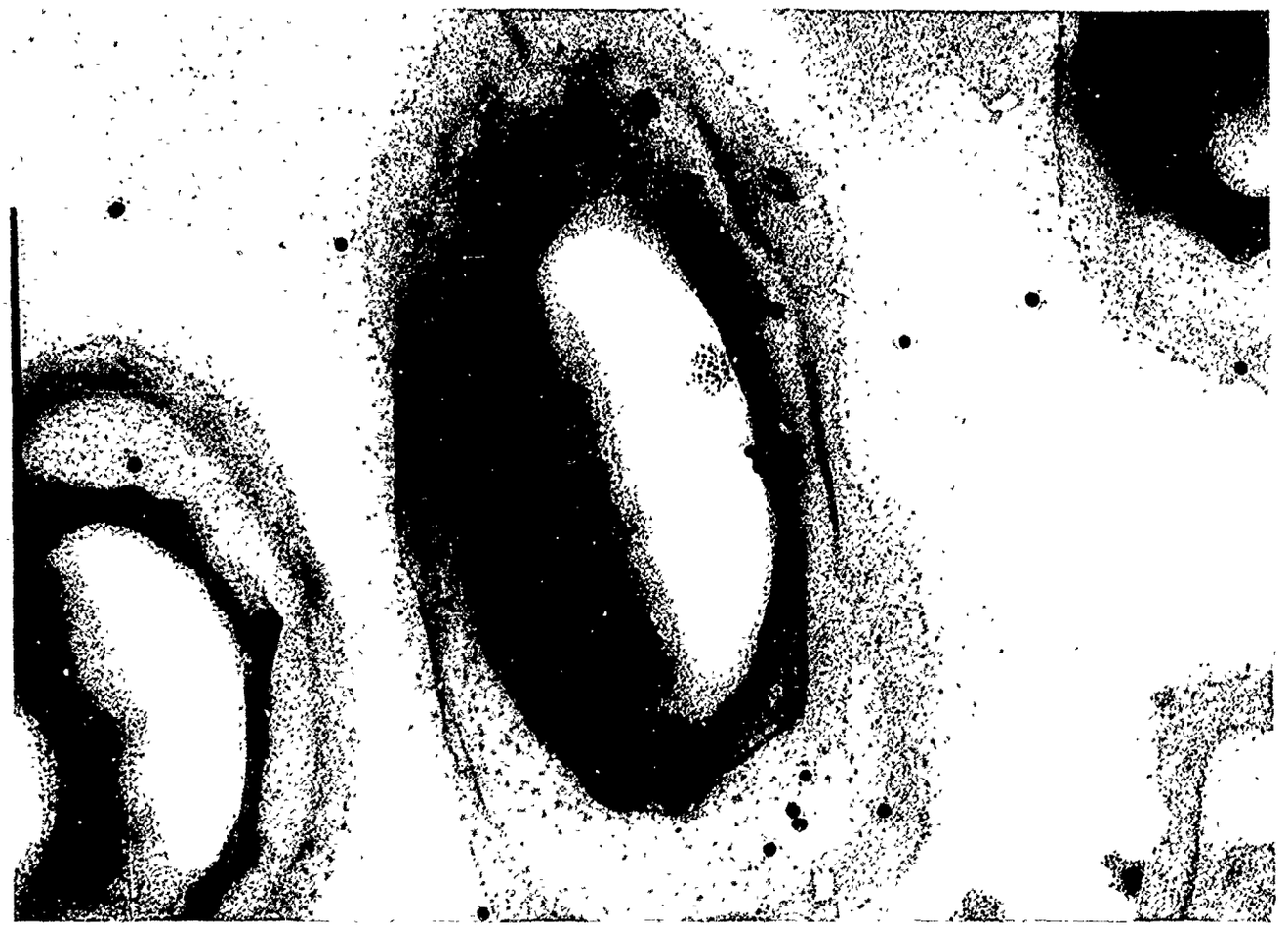
A very distinct and structurally clear representation of a mature, B.anthracis Sterne spore. The outer boundary is the exosporium. It seems like a delicate integument, draped around a rigid spore body. Spore coats form the next inner layer, followed by the cortex and finally the dense, inner core or the germinal layer. Treated with MAb to EA (1EAII-2C2-1-1). Mag.= 117,000

Fig. 69



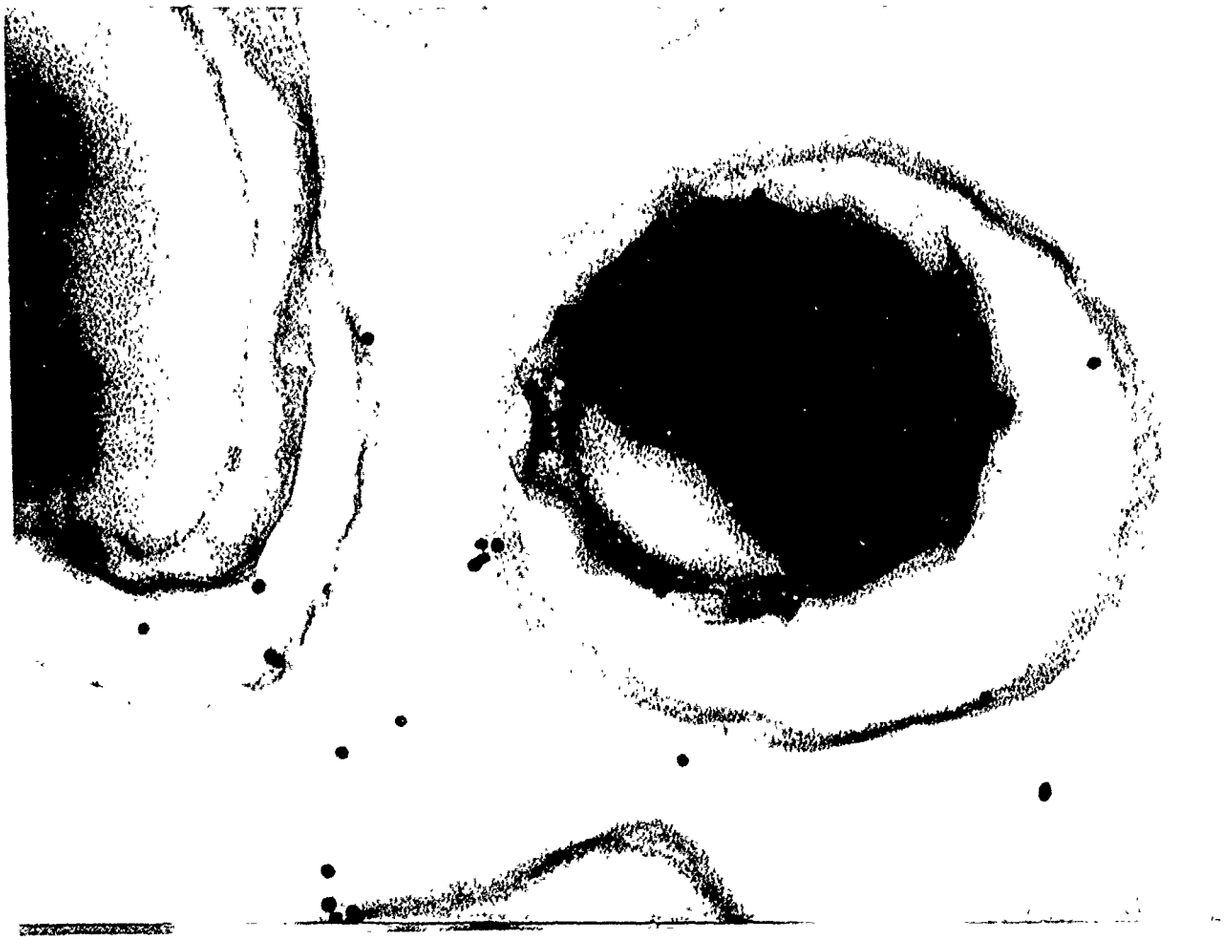
B. anthracis Δ-Sterne spore, unboiled and treated with anti-EA, 1EAII-2C2-1-J. No conclusions could be made regarding the presence of the EA antigen, based upon these studies. Mag.= 42,000

Fig. 70



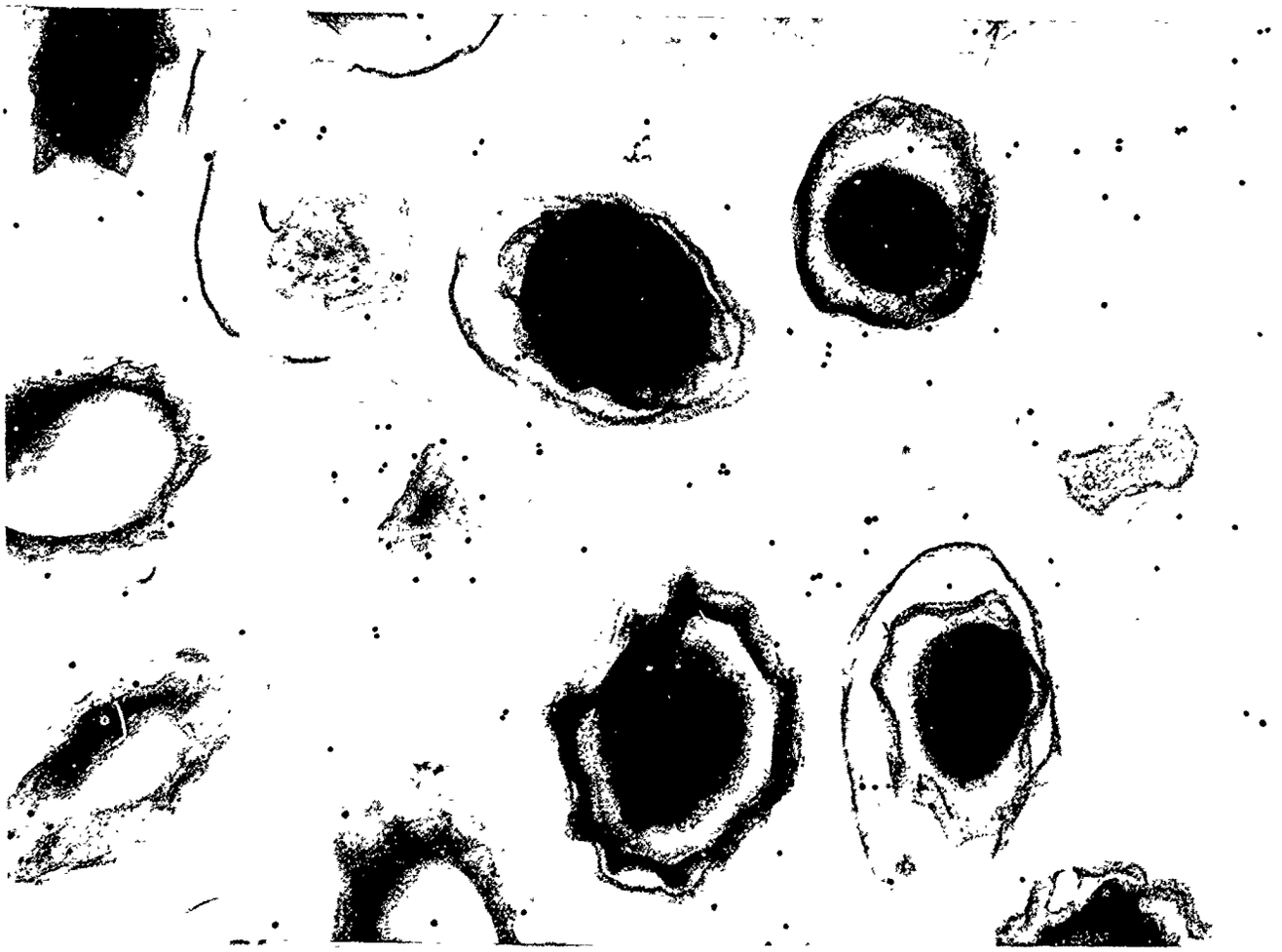
B.anthracis Δ-Sterne spore, unboiled and treated with anti-EA.
Mag.= 95,000

Fig. 71



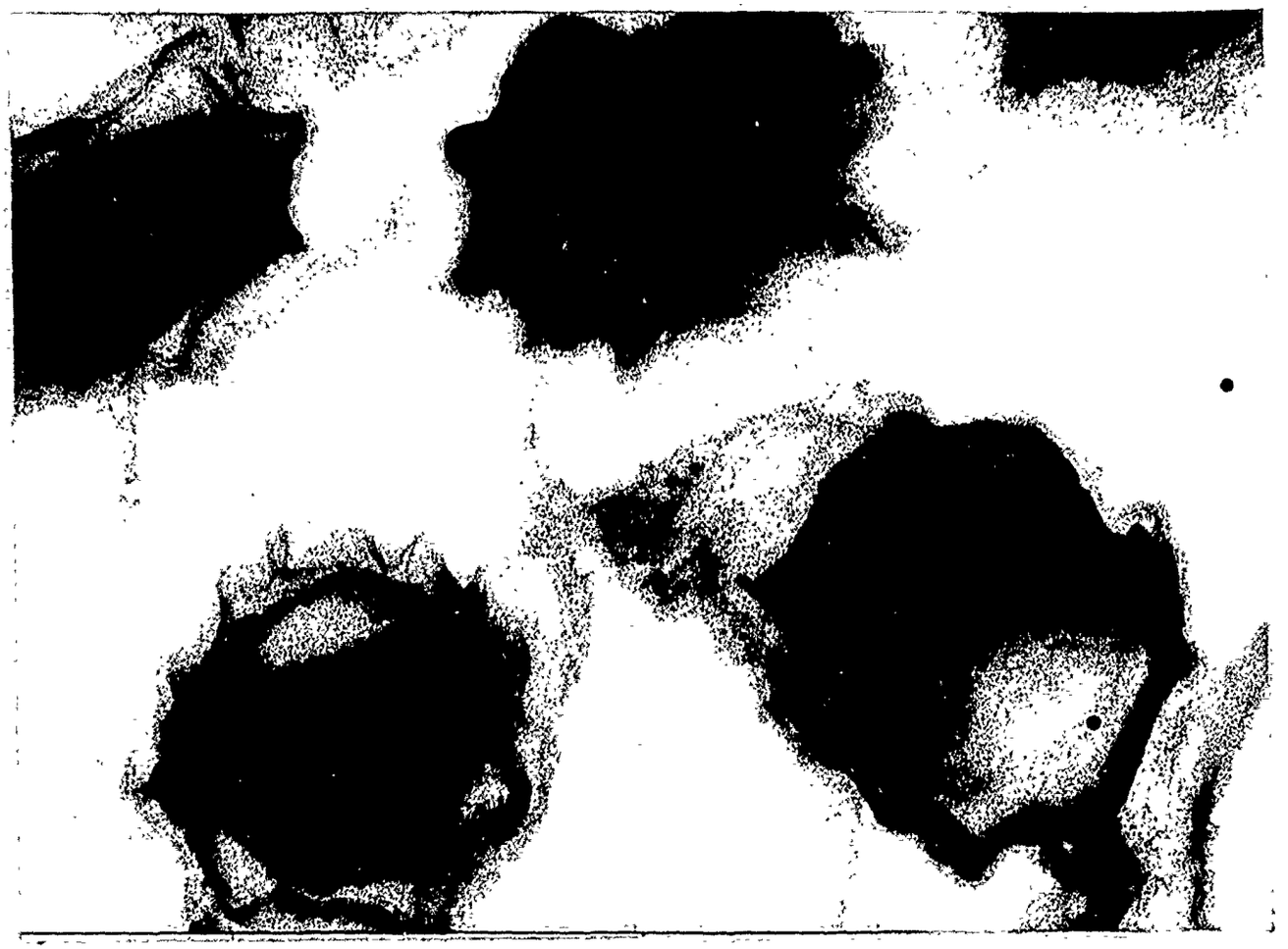
B.anthracis Sterne spores boiled for 15 minutes before exposing to the MAb to EA (1EAII-2C2-1-1). Mag.= 99,000

Fig. 72



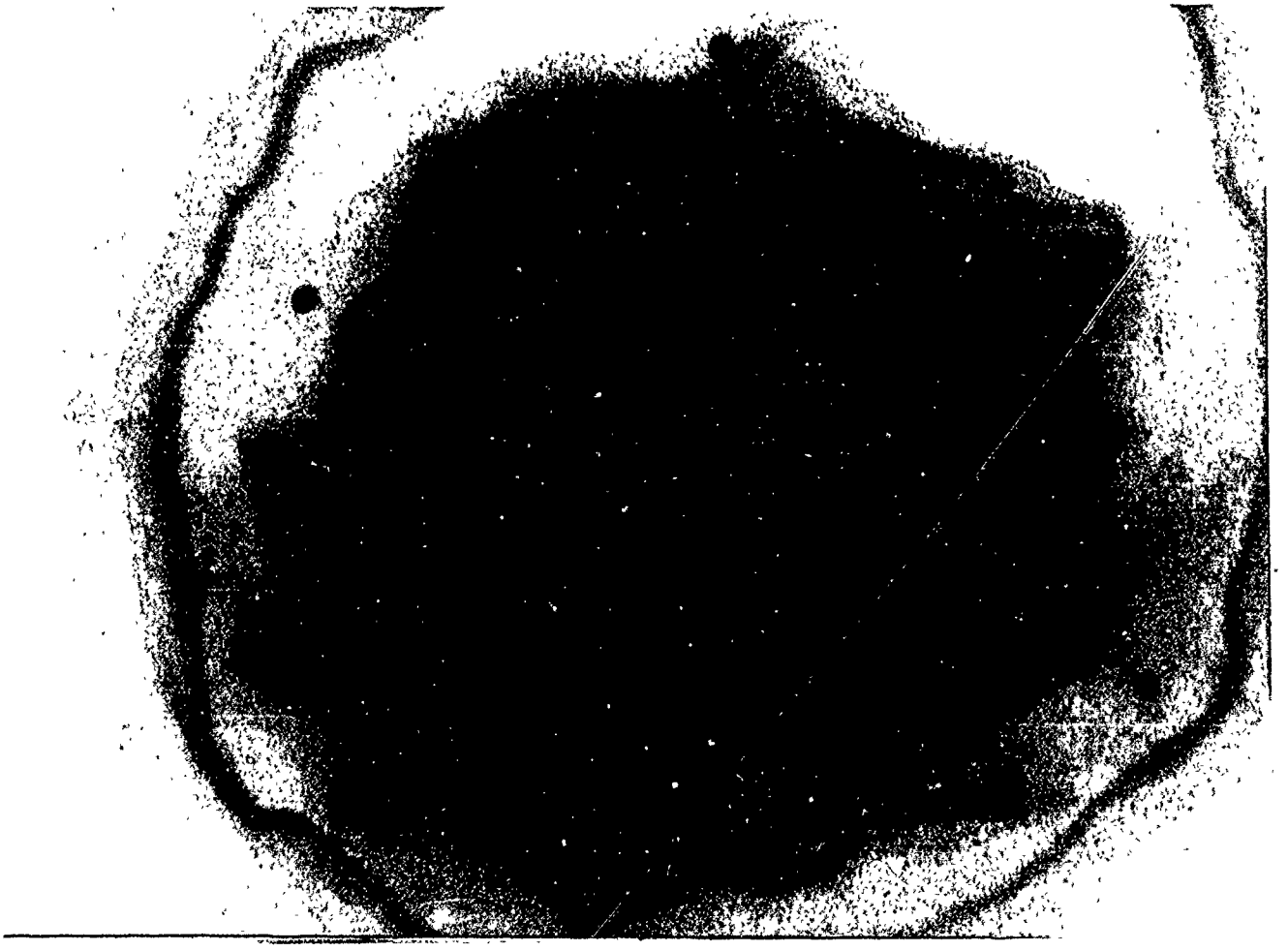
B.anthracis Sterne spores in various stages of development. Boiled and treated with 1EAII-2C2-1-1, a MAb against the extractable antigen. Mag.= 65,000

Fig. 73



B.anthracis 4229 spores treated with MAb to PA. Note the more or less random labelling. No conclusions could be made based upon these observations. Mag.= 95,000

Fig. 74



B.anthracis Δ-Sterne spore, boiled and treated with PA1-107-2-1. The labelling is random and scattered. Mag.= 210,000

Fig. 75

B. anthracis Sterne spores, boiled for 15 minutes and exposed to MAb to PA (PA1-1G7-1-2) Labelling is random and scattered.
Mag.= 63,000

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EXTRACTION OF B. ANTHRACIS CELLULAR PROTEINS:-

Sterne, Δ-sterne and 4229 strains of the organism were subjected to the protein extraction procedure of Ezzell and Abshire (Infection and Immunity, 56, 1988); see section entitled "Protocols".

The concentrated protein ex were subjected to:

[A] ELISA - using the M s to EA.
[B] SDS-Polyacrylamide Gel Electrophoresis.
[C] Western Blots

[A] ELISA

Aim:-

(i) To ascertain whether the protein extracted from B. anthracis Sterne cells does in fact contain the extractable antigen (EA). (ii) Does this protein react specifically with the monoclonal antibodies (1EAI-8D8 and 1EAII-3F 11) supplied by the Army.

Materials:-

PBS, PBST (PBS + 0.3% Tween 20), MAbs to EA 1EAI-8D8 and 1EAII-3F11, Protein A-HRP, Anti Ig-HRP, o-phenylenediamine, hydrogen peroxide, citric acid, Na_2HPO_4, 2.5 M H_2SO_4.

Method:-

The concentrated Sterne protein extract was diluted into 2 fold serial dilutions using PBST. 50 ul of each dilution was placed in respective wells of a 96 well microtiter plate and incubated for 2 hr at 37°C. The plate was washed 3 times with PBST. The monoclonal antibodies (MAbs) were diluted 1:50 using PBST. 50 ul of each of the monoclonals was added to the wells and incubated for 1 hr at 37°C. The control wells did not receive the MAb, only PBST. The plate was washed again with PBST. 50 ul of a 1:500 diluted Protein A-HRP or Anti Ig-HRP were added to the washed wells and incubated for an additional hour. To develop the color, 100 ul of a substrate buffer (12.5 ml 0.1 M citrate + 12.5 ml 0.1M Na_2HPO_4 + 20 mg OPD + 25 ul H_2O_2) were added and the plate incubated in the dark for 30 min. The reaction was stopped with 50 ul of a 2.5 M H_2SO_4 solution.

Observations:-

TABLE 1

MAb	HRP Conjugate	1	2	3	4	5	6	7	8	9	10	11	12
1EAI-8D8	Anti-Ig HRP	1.82	1.42	1.57	1.43	1.25	1.16	0.27	0.47	0.53	0.76	0.04	0.03
	Protein A-HRP	1.57	1.13	1.14	1.84	0.6	0.64	0.05	0.08	0.12	0.04	0.01	0.01
	Control	0.21	0.19	0.20	0.18	0.18	0.19	0.20	0.21	0.18	0.20	0.18	0.21
1EAII-3F-11	Anti-Ig-HRP	1.40	1.44	1.4	1.29	1.01	1.01	0.45	0.32	0.47	0.42	0.04	0.03
	Protein A HRP	1.20	1.35	0.75	0.74	0.75	0.39	0.58	0.77	0.42	0.16	0.20	0.10
	Control	0.20	0.17	0.21	0.26	0.17	0.20	0.18	0.21	0.27	0.18	0.17	0.19

Results:-

The protein that was extracted from the cells of B. anthracis was able to give a strong color response to EA monoclones when combined in the ELISA technique. The table above demonstrates this quite clearly.

[B] SDS-Polyacrylamide Gel Electrophoresis:-

6 to 14% SDS-PAGE gels were run using the extracted cell proteins. A 10% gel seemed to be the best in terms of resolution and separation of the bands (See section on "Protocols"). Gels were routinely stained by either Coomasie Blue or the Silver stain. An array of distinct bands appeared in the vicinity of 60K, 80K and 90K. [Figs 76 & 77 are representative gels. The first is Coomassie stained. B. anthracis Sterne protein was used in conjunction with the molecular weight standards to see a general profile. The second has been silver stained.] In order to differentiate among the bands, it was essential to run Western Blots.

[C] Western Blots:-

The Western Blot analyses allows one to identify the specific proteins that have been resolved by SDS polyacrylamide gel electrophoresis, by binding with specific

antisera. Proteins resolved on an acrylamide gel are transferred to an NC filter that is incubated with the antisera. The primary antibody specifically binds its epitope and the bound antibody is detected with a secondary species, such as HRP-Protein A or HRP-anti Ig.

Materials:-

REAGENTS

1. TRANSBLOT BUFFER

Tris (THAM), 0.025 M	9.09 g
Glycine, 0.193 M	43.5 g
Milli-Q dH_2O	2400 ml
(Adjust pH to 8.35 without methanol)	
Methanol	600 ml
(De-gas under house vacuum for 10 min)	

2. PBS

(10 mM Monobasic Na phosphate + 0.85% NaCl)

	Amount/liter
NaH_2PO_4 ($NaH_2PO_4 - 2H_2O$)	0.311 g (0.35 g)
Na_2HPO_4 ($Na_2HPO_4 - 7H_2O$)	1.06 g (2.01 g)
NaCl	

(adjust pH to 7.3 with 1 M NaOH)

3. QUENCH BUFFER

Gelatin	0.5 g
Na Citrate	0.882 g
Non-fat Powdered Milk	5.0 g
PBS to 100 ml	

(Add gelatin to PBS 1st, warm in microwave to dissolve. Do not use excessive heat. Adjust pH to 7.5. Add citrate and milk.)

4. PBST (0.3% Tween)

PBS	100 ml
Tween 20	300 ul

5. PBSTG

PBST	100 ml
Gelatin	0.5 g

Secondary Antibody

Make according to experimental instructions (dilute in PBSTG).

For example:
Rabbit anti-mouse IgA, IgG, IgM (Calbiochem-Behring Corp., Catalogue #574901), 1:400 in PBSTG.

Horseradish-peroxidase (HRP) conjugates

Rabbit anti-goat-HRP (Kirkgaard & Perry)
Dilute 1:600 in PBSTG

Protein-A-HRP (Dr. Leppla, USAMRIID)
Dilute 1:5000 in PBSTG.

TETRAMETHYL-BENZIDINE REAGENT

1.	Tetramethyl-benzidine 5 mg/ml methanol (heat gently to dissolve)	5.0 ml
2.	HEPES 1.0 M, pH 7.5	1.25 ml
3.	Milli-Q dH_2O to 100 ml Immediately prior to use add 25 ul 30% H_2O_2.	
4.	Dioctylsodium- sulfosuccinate 1.0%	20.0 ml

Add the above in sequence.

STORAGE BUFFER

HEPES 1.0 M, pH 7.5	3.0 ml
Dioctylsodium- sulfosuccinate 1.0%	60.0 ml
Methanol	15.0 ml
Milli-Q dH_2O to 300 ml	

Method:-

Following electrophoresis, the acrylamide gel was removed from the glass sandwich of vertical gel electrophoresis chamber. Whatman #3 filter-paper (4 sheets) and nitrocellulose (NC) paper were precut to the size of the gel to be transblotted. Since NC was to be cut into strips, the back of the paper was marked with a line about 5 mm from the bottom for subsequent lining up of the strips. The NC was also marked 2.5 mm apart in order to cut into strips after transblotting.

The sponges of the transblot chamber, filter paper and NC were prewet in Transblot Buffer. Two pieces of filter paper were placed on the sponge, the gel was placed on top of the filter paper in the reverse orientation of the

original run. The prewetted NC sheet was carefully placed on the gel so as to prevent air bubbles from becoming entrapped. (Essential to wear gloves when handling NC). Two wet sheets of filter paper were layered on top of the NC. The support was then lowered into the transblot chamber while insuring that the NC side was facing the positive side (red terminal) of the chamber. The chamber was filled with the transblot buffer, the terminal leads were connected appropriately and the amperage set at 200 mA and run for 5 hours in the cold room. The buffer was constantly stirred with a magnetic stir bar.

The NC was carefully removed from the support, and frozen for future use in Quench buffer containing 50% glycerol. When needed, 2.5 mm strips were cut and placed in Accutran trays (Schleicher and Schuell, Inc.) and washed 3 times with PBST. The plate was covered with plastic film in order to prevent evaporation during incubation. They were incubated at 37°C for 2 hr in specified antibody (diluted in PBSTG) using 1 ml per Accutran well. The NC strips were then washed 4X with 1 ml/well of PBST, 3 min/wash.

For goat, rat and mouse sera or mouse monoclonals, the strips were incubated for 30 min at room temperature in 1 ml of the appropriate Ab-HRP conjugate diluted in PBSTG. If human, guinea pig or rabbit Ab was used, then protein-A-HRP (from Leppla, USAMRIID) diluted 1:5000 in PBSTG was used.

The strips were washed 4 times in PBST. Tetramethyl-benzidine reagent at 1 ml/strip was added to each well of the tray. The reaction was stopped after 10 min by washing once with distilled water and once with the storage buffer at 5 min per wash. A positive response is indicated by the appearance of a bluish green band. The strips could be aligned on glass plates using the previously drawn line at the bottom and photographed from above.

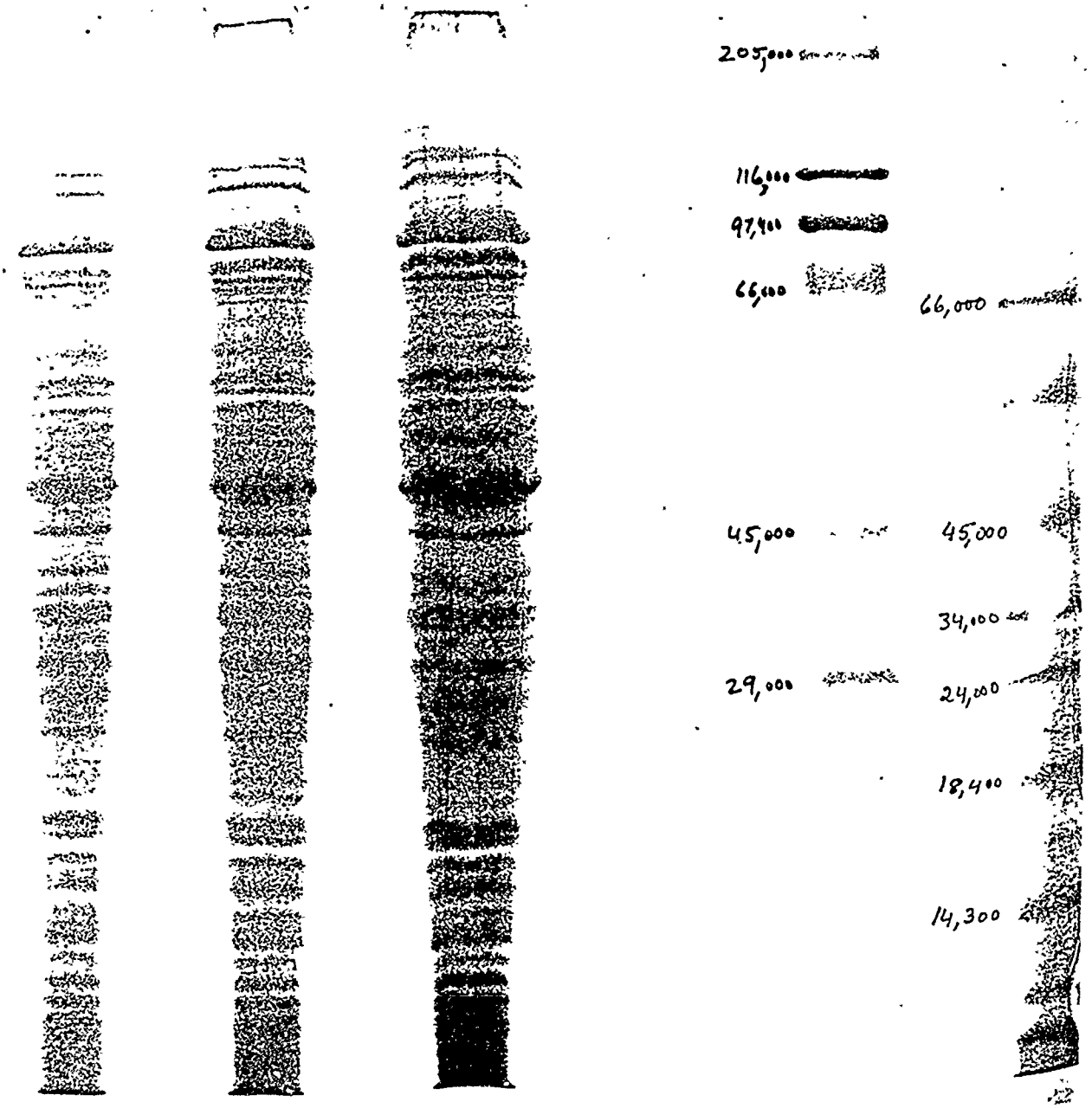
A mixture of the molecular mass protein standards was run in lanes adjacent to those containing cell extracts. Before the gel was placed on the transblotter, a small portion was cut off longitudinally and stained separately with either Coomassie Blue or the Silver stain (BioRad). The stained and dried gel was then lined up with the strips of the developed NC paper. The electrophoretic mobilities of the standard proteins were calculated and the molecular masses of the unknown proteins interpolated from the standard bands.

Results:-

The Western Blots revealed several bands (Fig 78). The molecular weight ranges appear to be consistent with those reported by Ezzell and Abshire (Infec. & Imm., Vol 56, 1988). Distinct bands showed up near the 90,000 mark when

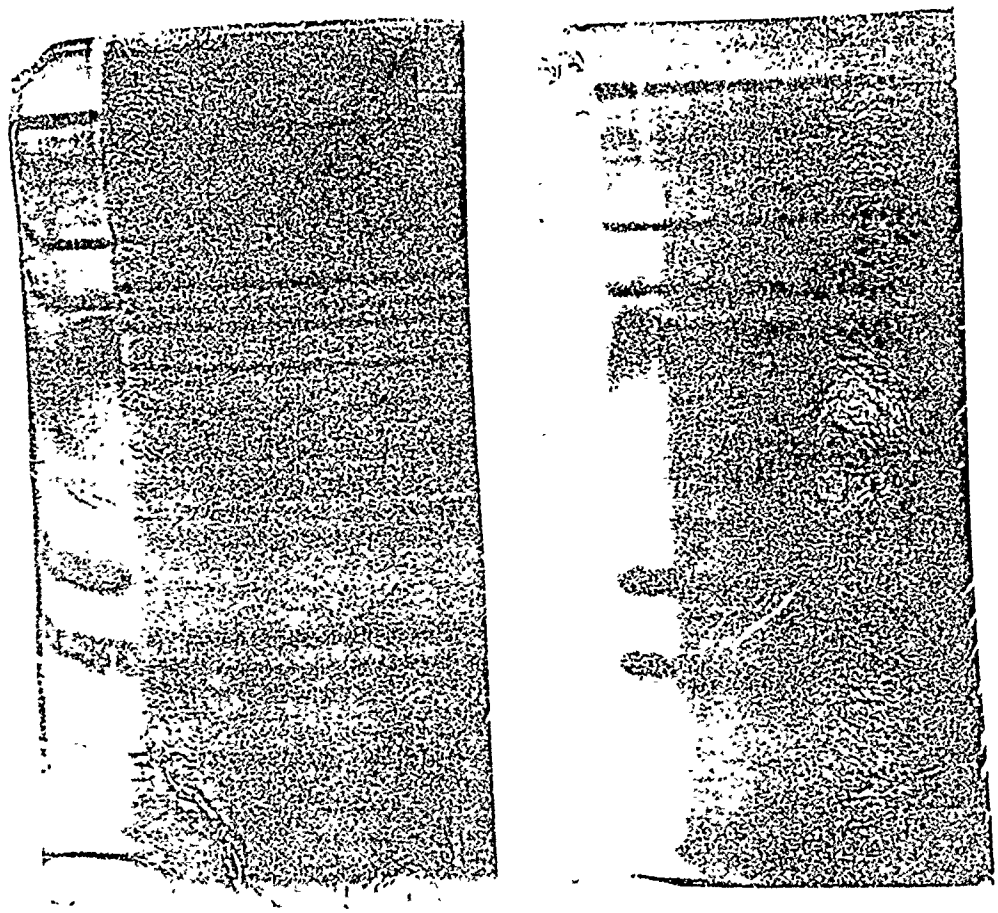
the NC paper was treated with the MAb to EA, EAII-1F6 (tube #127), and when the same sample of NC paper was tested with the various MAbs to PA, a prominent, wide band appeared consistently in the 85,000 range. Several breakdown products of PA also stained positively and appeared in the 50,000 area (Fig 79). For the above study, goat anti-mouse Ig, conjugated to horseradish peroxidase was used as the second antibody. When sera from AP-Anvax, GP-MDPH, Ezzell or Baker were employed, Protein-A-HRP was used as the second antibody.

FIG. 76



B. anthracis Sterne cell proteins. Extracted with 1% SDS/5mM mercaptoethanol. Stained with Coomassie Blue. Note size markers in right lane.

FIG. 77

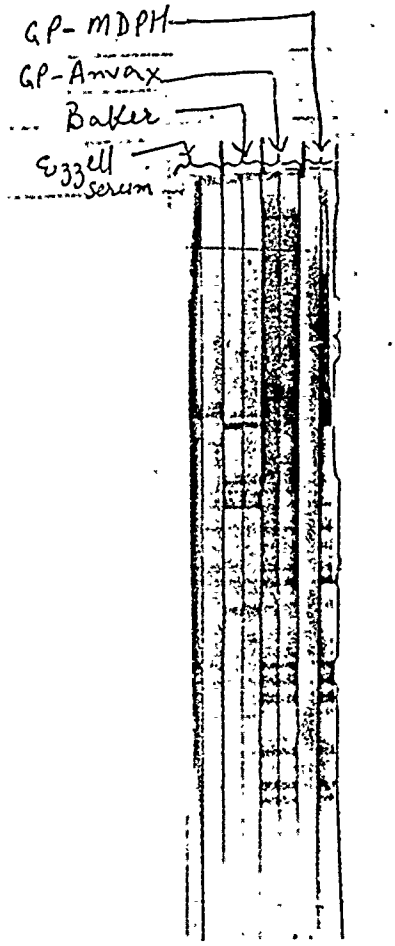


[SILVER STAINED GELS]

B. anthracis 4229
cell extracted protein.
100 µl (1:1 diluted in sample buffer).
10 µl M. Wt. Standards.

B. anthracis [illegible]
cell extracted protein.
100 µl (1:1 diluted in sample buffer)
10 µl M. Wt. Standards.

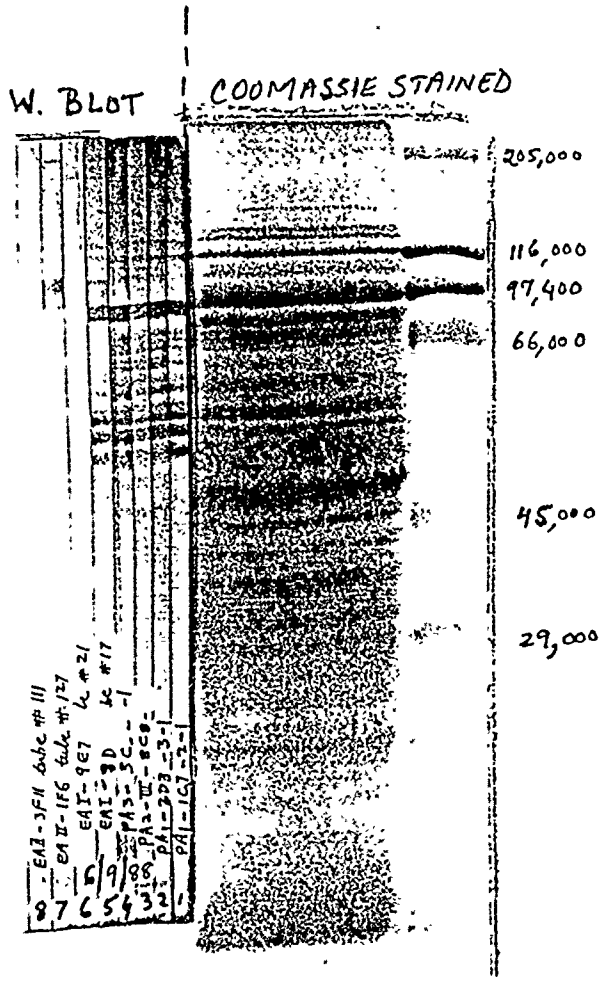
FIG. 78



Western Blot using the transblotted B. anthracis Sterne cell proteins in conjunction with the above sera.

FIG. 79

EA_1 = 91,500
PA = 85,430
LF = 82,000
EA_2 = ~ 60,000



B. anthracis Sterne cell proteins. Portions of the same gel are presented here. On left is the immunolabelled western blot. On the right is the Coomassie stained gel for comparison.

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ISOLATION, PURIFICATION, AND CHARACTERIZATION OF CELL WALL POLYSACCHARIDES OF *BACILLUS ANTHRACIS* (Δ-STERNE)

MATERIALS AND METHODS:-

Bacterial Strain

Bacillus anthracis (Δ-Sterne) was supplied by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) (Fort Detrick, Maryland). Cells were then maintained on AK sporulation agar slants (BBL, Cockeysville, Maryland) in the cold ($0^{o}C$-$4^{o}C$).

Preparation of Cells Walls

B. anthracis (Δ-Sterne) cells were grown overnight at $37^{o}C$ in a 20 ml Penassay Broth (PAB) (Difco-Antibiotic Medium 3) contained in nephlos flasks. About 5-6 ml of cell culture were then transferred to 1-1.2 liter PAB in 2.8 liter baffled flasks. The transferred cells were grown to late exponential phase (200-250 klett units), and then harvested by centrifugation and washed 2 times in cold distilled water. The cells were disrupted with a sonicator (Sonics and Materials, Inc., Danbury, Connecticut, USA) and walls obtained as previously described by Doyle *et al* (3), and Brown (4).

The cell wall preparation was purified by sequential extraction in hot 1% (w/v) sodium dodecyl sulfate (SDS), and finally by several washes in water (Doyle *et al*, 5). The walls were then stored in a freeze-dried form.

Isolation and Purification of Polysaccharide from Cell Wall

One hundred mg of freeze-dried cell wall was treated in 20 ml 48-51% hydrofluoric acid (HF; reagent grade) at $4^{o}C$ for 18 hours. The preparation was then centrifuged at 24,000 x g for 10 minutes. One ml of supernatant was mixed with 5 ml absolute ethanol and allowed to stand in the cold for about 30 miutes. The mixture was centrifuged as above, and the precipitate was washed 3 times with absolute ethanol. The final product (polysaccharide) was left at room temperature for about 30 minutes in order for the alcohol to evaporate and was then dissolved in 1-2 ml cold distilled water. The dissolved material was freeze-dried and stored in a desiccator in the cold.

Testing and Purity of Isolated Polysaccharide

The polysaccharide was usually analyzed with an amino acid analyzer (Dionex D-300) to check for any contamination by cell wall muramic acid. Little or no muramic acid was

usually detected in the preparations. Polysaccharide was also assayed for neutral sugar using the anthrone method (6). A consistent micromolar amount of hexose per milligram polysaccharide from one batch of polysaccharide to another gave a good indication that the isolated polysaccharide materials were pure (7).

Analytical Methods

Total hexose and hexuronic acid in the isolated polysaccharide were determined by the anthrone method (6) and the method of Dische (8), respectively. Specific type of hexose (galactose) was then determined by a modified method of Strecker and Korkes (9), whereas galacturonic acid was determined by the Knutson and Jeanes method (10). Total phosphorus was determined using a modified method of Ehrlich and Telep (11). Amino sugars were analyzed using a single column methodology, sodium citrate eluants, and a ninhydrin detection system (570 and 440 nm detectors). A Dionex-D-300 amino acid analyzer equipped with a Dionex CP-3 programmer (for eluant concentration, time, and temperature changes) were employed for all runs. Free amino groups were determined by the method of Sanger (12). Samples (2 mg) for amino sugar analysis were first acetylated as described by Niedermeier (13), and then hydrolyzed in 4 M HCL for 8 hours at 100^{o}C in sealed tubes. Analyses were also carried out with the hydrolyzates of samples that had been acetylated and reduced with sodium borohydride as described by Taylor and Conrad (14). Amino sugar standards were similarly treated and run with each set of samples. Also, amino sugars were determined using a thin layer chromatography method of Rebers and Wessman (15). Molecular weight of the polysaccharide was determined by the reducing end sugar assay (16). Finally, polysaccharide samples were sent to Complex Carbohydrate Corporation (Athens, GA 30605, USA) for independent analyses. Analyses was done by gas liquid chromatography, using column 15 m DB-1, initial temperature 140^{o}C, 3 min., final temperature 240^{o}C.

Periodate Oxidation and Smith Degradation

Periodate oxidation of the polysaccharide was done according to the method of Kojima *et al* (17). Briefly, 1 mg of polysaccharide was oxidized in 35 mM $NaIO_4$ in 10 mM sodium acetate buffer (pH 4.5) for three days in the dark at 4^{o}C. At the end of this time, excess $NaIO_4$ was destroyed by the addition of a few milliliters of ethylene glycol. The oxidized sample was then dialyzed against distilled water for 24 hours to remove low molecular weight substances, and non-dialyzable material was freeze-dried (18). Part of the freeze-dried sample was then analyzed for neutral sugars, while another portion was hydrolyzed with 4 M HCL at 100^{o}C for four hours (for analysis of amino sugars). The remaining portion of the sample (about 10 mg) was dissolved

in 5 ml of distilled water. To this mixture was added 2.5 mg of sodium borodeuteride, and the reaction mixture maintained for 24 hours at room temperature. At the end of the reduction reaction, the reaction mixture was dialyzed against distilled water for 24 hours, and the non-dialyzable fraction freeze-dried (16,18). Part of the freeze-dried sample was again analyzed for neutral sugars by anthrone assay, while another portion was hydrolyzed as above and analyzed for amino sugars. Finally, the remaining portion of the sample was subjected again to reoxidation with $NaIO_4$ as above.

Thin Layer Chromatography of Hydrolyzates of Smith Degradation Product (SDP) and HF-extracted Polysaccharide, Quantitation of Alditol Derivatives by HPLC

Smith degradation product was hydrolyzed to obtain products which could then be identified and utilized in structural characterization of the HF-extracted polysaccharide. Acid hydrolysis was done according to the Pazur and Forsberg method (19). Two milligrams of the SDP was hydrolyzed first with 0.1 M HCL for 3 hours at 100°C in a boiling-water bath. Another 2 mg SDP was mildly hydrolyzed with 0.02 M HCL for 20 minutes at 100°C. Finally, 2 mg of the HF-extracted polysaccharide was hydrolyzed with 4 M HCL for 4 hours at 100°C. Analysis of the three hydrolyzate products was performed by thin layer chromatography, using the method of Rebers and Wessman (15). The various hydrolyzate products were detected using silver nitrate and sodium hydroxide reagents (19). Finally, the hydrolyzate products (glycerol, threitol) of SDP were quantitated using high pressure liquid chromatography. An anion exchange column (Dionex AS-6A, 5 micron , 15 cm long, and 4 mm internal diameter) was used for the alditol separation. The mobile phase was 0.15 M NaOH, and detection was by electrochemical means.

Chromium Trioxide Oxidation and ^{13}C-NMR Spectroscopy

Anomeric configuration of glycosidic linkages in the polysaccharide was determined by chromium trioxide oxidation using the Hoffman method (20), and by ^{13}C-NMR spectroscopy. ^{13}C-NMR analyses of the HF-extracted polysaccharide and the Smith degradation product were also performed. All ^{13}C-NMR spectra were recorded at 75MHz on a Varian XL-300 instrument. An acquisition time of 0.2s. was used with a PW=3.0, LB=6.0, D1=0.2 as other important parameters. Usually 30,000-50,000 acquisitions were made. Table 2 shows some of the techniques generally applied towards polysaccharide sequencing, and the structural information derived from such techniques.

TABLE 2

Techniques applied towards polysaccharide (pCHO) sequencing and structural information derived from such techniques

Technique	Structural information	Drawback
Nuclear magnetic resonance spectroscopy (NMR, ^{1}H or ^{13}C)	Anomeric configuration, monosaccharide composition monosaccharide sequence (arrangement) and conformation of pCHO	Non-detection of presence of most inorganic impurities. Large samples required. Some samples required long running periods
Periodate oxidation	Quantitation of sugars at the non-reducing terminus or periodate-sensitive position of pCHO	Overoxidation due to formation of tartronic acid half-aldehyde derivatives from hexuronic acid end groups. Underoxidation (incomplete oxidation) due to protection of vulnerable units by interresidue hemiacetal formation or hydrogen bonding
Smith degradation	Distribution of periodate-resistant sugar residues, composition and types of linkages within backbone chain of pCHO	Loss of sugar residues due to acid-hydrolysis, acid-catalyzed transacetylation leading to formation of cyclic acetals

TABLE 2 (continued)

Techniques applied towards polysaccharide (pCHO) sequencing and structural information derived from such techniques

Technique	Structural information	Drawback
Methylation	Positions of glycosidic bonds, types of sugar residues	Incomplete methylation of some polysaccharides. No satisfactory means of monitoring completeness of methylation
Partial acid hydrolysis	Sequences of sugar residues; small oligosaccharides obtained make for easier resolution of pCHO structure	Oligosaccharides isolated are dependent on relative rates of cleavage without decomposition of glycosidic linkages

Adapted from The Polysaccharides (Vol. 1), edited by Aspinall, G.O. (4).

Results:-

Table 3 shows the composition of the HF-extracted polysaccharide. Two sets of data were obtained, one from independent analysis done by a Complex Carbohydrate Corporation in Athens, Georgia. Gas liquid chromatography was employed for the independent analyses of the polysaccharide (see analytical methods under materials and methods). The other set of data was obtained by using different analytical methods for the analyses of each monosaccharide residues contained in the polysaccharides (see analytical methods under materials and methods). The rationale was to come up with molar ratios of the monosaccharide residues that best complement the results obtained with ^{13}C-NMR spectroscopy. Analyses done by the independent carbohydrate laboratory showed that the polysaccharide was composed of galactose, N-acetylglucosamine and N-acetylmannosamine in molar ratios of 3:2:1, respectively. Galacturonic acid was not detected. When the polysaccharide was analyzed by different chemical methods, it was shown to contain galactose, N-acetylglucosamine, N-acetylmannosamine and galacturonic acid in molar ratios of 2:1:1:0.2, respectively. However, ^{13}C-NMR spectrum of the polysaccharide (Fig. 8D) showed the presence of 6-7 anomeric (C-1) carbon atoms which corresponded to molar ratios of 3 galactose, 2 N-acetylglucosamine, 1 N-acetylmannosamine and 0.2 galacturonic acid. A thin layer chromatogram of the polysaccharide hydrolyzate confirmed the presence of galactose, N-acetylglucosamine and N-acetylmannosamine in in the polysaccharide, but galacturonic acid again was not detected (Fig. 8I). So the presence of galacturonic acid in the polysaccharide was confirmed by chemical methods only. Table 4 shows average yields of polysaccharide per 100 mg dry cell wall obtained at different hydrolysis conditions. Results indicated that 18h HF-hydrolysis was optimal for isolation, since the amount of polysaccharide was highest in this case. Ethanolic precipitation appeared to give higher yield than dialysis against distilled water. This could be seen when the 18h HF-polysaccharide yield obtained via ethanolic precipitation and that obtained through dialysis were compared (twice as much of the polysaccharide obtained via dialysis was given by ethanolic precipitation). The low yield obtained following 24h HF-hydrolysis and ethanolic precipitation might have been due to human error during fractionation. In all cases, however, irrespective of the hydrolysis condition and means of recovery, the polysaccharide components remained the same (Table 5).

Sodium Periodate Oxidation and Smith Degradation

Table 6 shows the results of sodium periodate oxidation and Smith degradation. Approximately 93% of the galactose residues were destroyed by periodate treatment. Also, about

50% of the galacturonic acid were sensitive to periodate treatment, whereas the two amino sugars (N-acetylglucosamine and N-acetylmannosamine) were resistant to periodate oxidation. This implies that the galactose residues were either at the non-reducing end of the polysaccharide or at the periodate-sensitive position within the polysaccharide.

Following Sodium periodate oxidation and dialysis, the non-dialyzable fraction was reduced with sodium borodeuteride. The reduced product was reoxidized with sodium periodate and analyzed. Results (Table 6) showed that all sugar components were recovered in the same molar ratio as was obtained following the first-periodate oxidation. This implies that the polysaccharide moiety is made up of a linear backbone chain of galactose, N-acetylglucosamine, and N-acetylmannosamine, and these backbone residues may have been linked by 1->3 or 1->4 glycosidic bonds. All the amino sugars appeared to be internally linked; hence, they were resistant to periodate treatment. The galacturonic acid may be attached as a side chain to the backbone.

Thin Layer Chromatography of Hydrolyzates of Smith Degradation Products and HF-Extracted Polysaccharide

Figure 82 shows expected end-products of reaction of sugar residues at non-reducing ends of internally linked by 1,2, 1,4 or 1,6 bonds following Smith degradation. Figure 83 shows the results of Smith degradation product (SDP) hydrolyzed with 0.1 M HCL for 3 hours at 100°C (Lane 2). Lane 3 shows the result obtained when the HF-extracted polysacharide was hydrolyzed with 4 M HCL for 4 hours at 100°C. The chromatogram of the SDP showed that galactose was absent, whereas the two amino sugars (N-acetylglycosamine and N-acetylmannosamine) were present in high proportions. In contrast, in the hydrolyzate of the HF-extracted polysaccharide (Lane 3), both galactose and the two amino sugars were present in high amounts. Also, glycerol and threitol were liberated from the SDP as shown in Lane 2. When the SDP was mildly hydrolyzed with 0.02 M HCL for 20 minutes at 100°C, only glycerol was produced (Lane 4). Glycerol is supposed to be liberated either from non-reducing terminal galactose residue or from the internal galactose residue linked through positions 1 and 6 or 1 and 2 (Fig. 82). Threitol, on the other hand, would result from the internal galactose linked through positions 1 and 4 (Fig. 82). Since both glycerol and threitol were liberated from the SDP, some of the galactose residues were either at the non-reducing terminus or linked through positions 1 and 2 or 1 and 6, while some were linked by either 1,3 or 1,4 bonds. The amounts of glycerol and threitol produced from the hydrolyzate product of SDP were determined by high liquid pressure chromatography and the molar ratio of glycerol to threitol was shown to be roughly 1:1. This

molar ratio is supposed to correspond to the number of 1,4 and 1,6 bonds involved in the linkage of galactose molecules.

^{13}C-NMR results showed that only two types of linkages 1,4 and 1,6 were found in the HF-extracted polysaccharide. This observation thus eliminates the presence of either 1,2 or 1,3 bonds in the polysaccharide. The HF-extracted polysaccharide appeared to contain a repeat unit of three galactose molecules linked by 1,4 and 1,6 glycosidic bonds, two N-acetylglucosamine and one N-acetylmannosamine, each linked by 1,4 glycosidic bonds.

Determination of Anomeric Configurations

Anomeric configurations of the glycosidic linkages in the HF-extracted polysaccharide was determined using chromium trioxide oxidation. Results showed that the galactose residues were β-linked, while the amino sugars appeared to be α-linked. These results were confirmed by ^{13}C-NMR which showed the presence of two α and two β, plus two α or β anomers (Fig. 80).

TABLE 3

Composition of hydrofluoric-acid extracted polysaccharide

Component	Amount (μmoles/mg pCHO)	Molar Ratio	Independent Analysis Amount (μmoles/mg pCHO)	Independent Analysis Molar Ratio
Muramic acid	0.00		0.00	
Glutamic acid	0.00		0.00	
Alanine	0.00		0.00	
Diaminopimelic acid	0.00		0.00	
Galactose	1.70	1.9 (2)	2.02	2.9 (3)
N-Acetylglucosamine	1.32	1.4 (1)	1.37	1.9 (2)
N-Acetylmannosamine	0.90	1.0 (1)	0.69	1.0 (1)
Total hexose	1.70		2.02	
Galacturonic acid	0.20		-	

TABLE 4

Preparation of Δ Sterne polysaccharide

Hydrolysis condition	Method of recovery	Average yield (mg) per 100 mg of dry cell wall
18h HF-treatment at 4°C	Ethanolic precipitation	18
24h HF-treatment at 4°C	Ethanolic precipitation	9
30h HF-treatment at 4°C	Ethanolic precipitation	13
18h HF-treatment at 4°C	Dialysis against distilled water	9

TABLE 5

Composition of polysaccharide isolated under different hydrolysis conditions

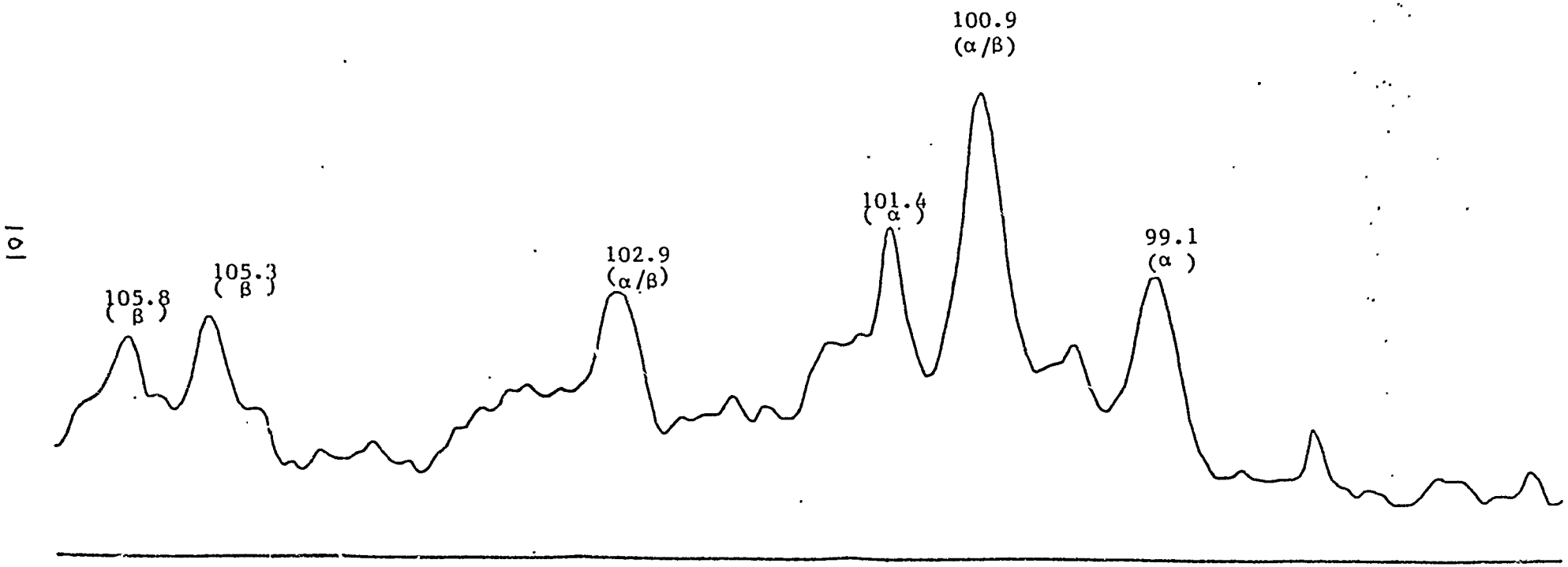
Hydrolysis condition	Component	Amount (μmoles) present in 1 mg polysaccharide
18h HF-hydrolysis	D-galactose	1.70
	N-acetylglucosamine	1.32
	N-acetylmannosamine	0.90
	D-galacturonic acid	0.20
24h HF-hydrolysis	galactose	1.70
	N-acetylglucosamine	1.32
	N-acetylmannosamine	1.02
	galacturonic acid	0.20
30h HF-hydrolysis	galactose	1.70
	N-acetylglucosamine	1.50
	N-acetylmannosamine	1.02
	galacturonic acid	0.20

TABLE 6

Results of sodium periodate oxidation

Component	Amount (μmoles) per mg pCHO	Amount (μmoles) per mg pCHO after periodate oxidation	Amount (μmoles) per mg pCHO after sodium-borodeuteride reduction and periodate reoxidation
Galactose	2.02 (3.0)	0.5	0.4
N-acetylglucosamine	1.37 (2.0)	2.2	2.6
N-acetylmannosamine	0.69 (1.0)	1.7	1.8
Galacturonic acid	0.20 -	0.1	0.1

FIGURE 80



^{13}C-NMR spectrum of anomeric (C-1) carbon atoms of the HF-extracted polysaccharide.

FIGURE 81

Thin layer chromatography of PCHO hydrolyzate.

Lane 1: Mixture of standards

Lane 2: PCHO hydrolyzate

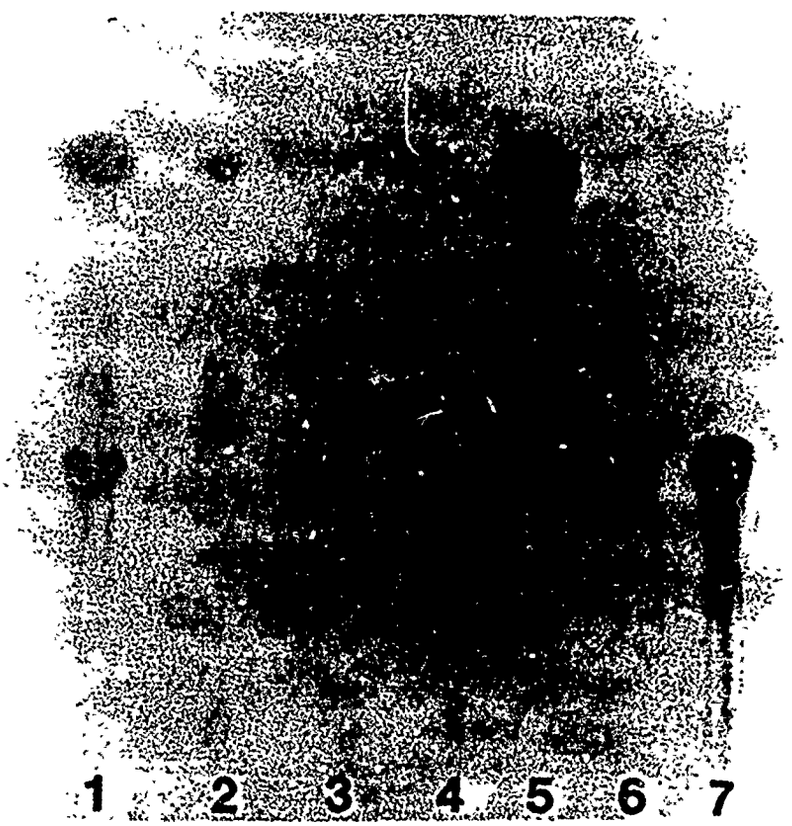
Lane 3: Glucosamine standard

Lane 4: Mannosamine standard

Lane 5: Galactose standard

Lane 6: Galactosamine standard

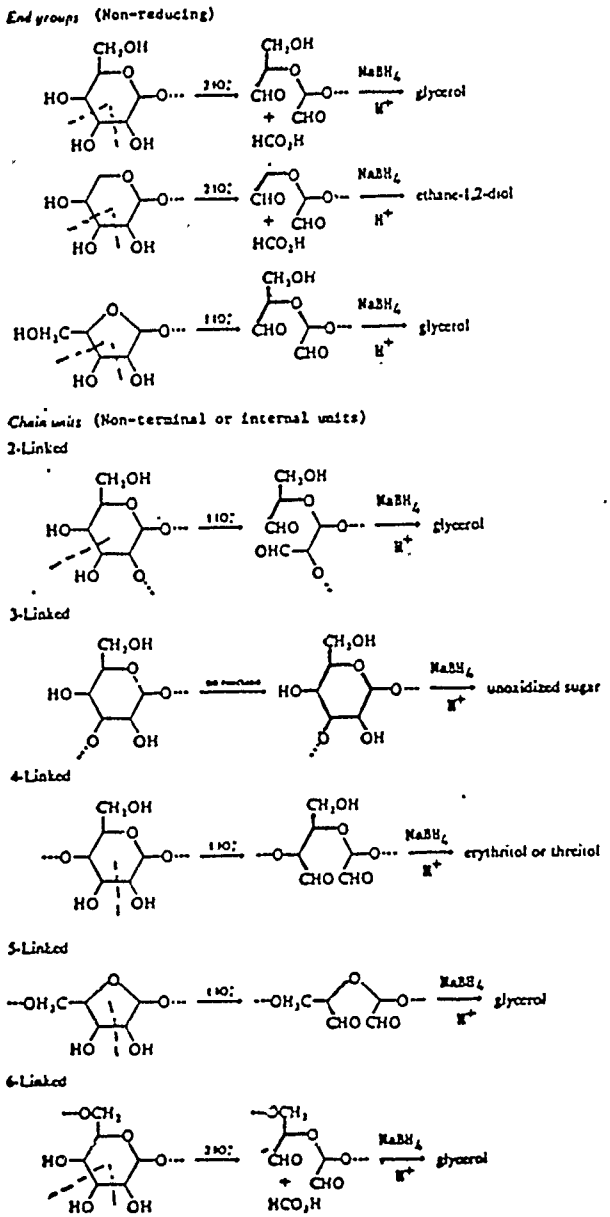
Lane 7: Galacturonic acid standard



THIN LAYER CHROMATOGRAPHY OF PCHO HYDROLYZATE

Lane 1: Mixture of standards
Lane 2: PCHO hydrolyzate
Lane 3: Glucosamine standard
Lane 4: Mannosamine standard
Lane 5: Galactose standard
Lane 6: Galactosamine standard
Lane 7: Galacturonic acid standard

FIGURE 32.



Reaction of variously linked sugar residues with periodate indicating, where appropriate, moles of oxidant consumed and one-carbon fragments liberated, and products formed from those portions of the original sugar units shown in heavy print on reduction with sodium borohydride followed by complete hydrolysis. (The ---- indicate points of periodate attack.) Adapted from The Polysaccharides, (Vol. 1), edited by Aspinall, G.O. (21)

FIGURE 83

Thin layer chromatography of hydrolyzates of Smith degradation product and HF-extracted polysaccharide.

Lane 1: Glycerol standard

Lane 2: Smith degradation product hydrolyzed with 0.1 M HCL for 3 hours at 100°C

Lane 3: HF-extracted polysaccharide hydrolyzed with 4 M HCL for 4 hours at 100°C

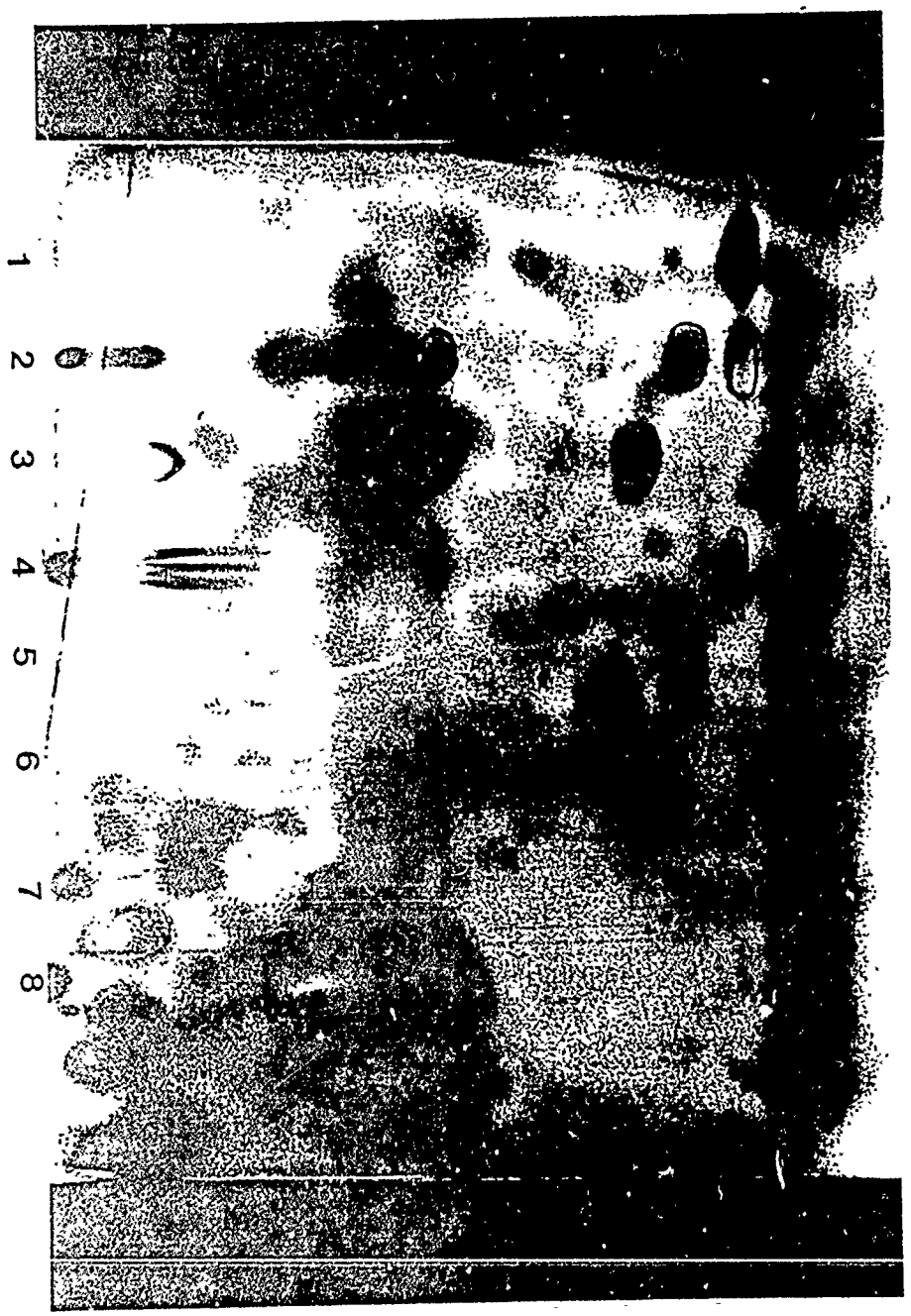
Lane 4: Smith degradation product hydrolyzed with 0.02 M HCL for 20 minutes at 100°C

Lane 5: Threitol standard

Lane 6: Galactose standard

Lane 7: Glucosamine standard

Lane 8: Mannosamine standard



DISCUSSION

The results of this study show that a polysaccharide fraction from B. anthracis (Δ Sterne) cell wall has been isolated and purified to apparent homogeneity. Table 6 shows the composition of the HF-extracted polysaccharide. Two sets of data were obtained (Table 3): one set from analyses of the polysaccharide using different chemical methods to analyze each polysaccharide component. The second set of data (independent analysis) was obtained by analyzing all polysaccharide components by the same method (gas liquid chromatography). The rationale was to determine molar ratios of the monosaccharide residues that best correspond to the results obtained with the ^{13}C-NMR spectroscopy (Fig. 80). The polysaccharide is made up of galactose, N-acetylglucosamine, N-acetylmannosamine and glacturonic acid, and molar ratios of 3:2:1:0.2 are most consistent with the number of anomeric (C-1) carbons observed in the ^{13}C-NMR spectrum of the HF-extracted polysaccharide (Fig. 80). The spectrum also showed the presence of 1,4 and 1,6 bonds only, thus eliminating the presence of 1,2 or 1,3 bonds in the anthrax polysaccharide. Structural analyses of the polysaccharide indicated that the most likely structure of the repeating unit of the polysaccharide is: ->4)Galβ(1->6)Galβ(1->4)GlcNAcα(1->4)Galβ (1->4)GlcNAcα/β(1->4)ManNAc α(1->.
The galacturonic acid may be attached as a side chain to the backbone structure of the polysaccharide. The molecular weight of the polysaccharide as determined by the reducing end assay was shown to be about 15,000. The repeat unit structure is in agreement with the results of ^{13}C-NMR spectrum as shown in Figure 80. Sodium periodate oxidation led to the destruction of about 93% of t e galactose residues, implying that these residues were at the non-reducing end or a periodate-sensitive position of the polysaccharide (Table 6). The two amino sugars, on the other hand, were resistant to periodate oxidation. This indicates that both amino sugars may be internally linked by 1,3 or 1,4-bonds (1,3 bonds have been ruled out by ^{13}C-NMR). Hydrolysis of the sodium periodate oxidized and sodium borodeuteride reduced polysaccharide (Smith degradation) gave rise to glycerol, threitol, as well as the two amino sugars, while galactose was absent (Fig. 83, Lane 2). In contrast, the hydrolyzate of the HF-extracted polysaccharide yielded galactose and the two amino sugars (Fig. 83, Lane 3). This observation showed that periodate oxidation, followed by sodium borodeuteride reduction converted all the galactose residues to their alditol derivatives. These derivatives (glycerol and threitol) were then quantitated and utilized in further structural characterization of the polysaccharide. The amounts of glycerol and threitol produced from the hydrolyzate product of Smith degradation were determined using high pressure liquid chromatography.

The molar ratio of glycerol to threitol was shown to be roughly 1:1, and this corresponded to the number of 1,4 and 1,6 bonds involved in the linkage of the three galactose molecules in the HF-extracted polysaccharide. This shows that one of the three galactose molecules in the repeat unit is linked through positions 1 and 6, while another is linked through postions 1 and 4. The third galactose may be linked by either 1,4 or 1,6 bond. The amino sugars (N-acetylglucosamine and N-acetylmannosamine), on the other hand, are each linked by 1,4 bonds. Anomeric configurations were determined by chromium trioxide oxidation. The galactose residues were shown to be β-linked, while the amino sugars appeared to be α-linked. The chromium trioxide result was further supported by ^{13}C-NMR spectrum of the polysaccharide which showed the presence of two α, two β and two α/β configurations (Fig. 8Ø). The HF-extracted polysaccharide partly resembles the anthrax polysaccharide isolated by Mester et al (22,23), except for the presence of N-acetylmannosamine and galacturonic acid which are also components of the HF-extracted polysaccharide. The polysaccharide described by Mester et al (22,23) is known to contain N-acetylglucosamine linked in position 1,4 and D-galactose molecules linked in position 1,4 (or 1,2) and also in position 1,6 (or present in terminal groups). Anthrax polysaccharide is known to cross-react with type XIV pneumococcal antiserum (24,25). Mester et al (22,23) suggested that the cross-reaction might be due to the presence of multiple residues of 1,6-linked galactose in the anthrax polysaccharide. Finally, the HF-extracted polysaccharide does not contain glycerol or phosphorus, and as such is not a teichoic acid. The presence of a small amount of galacturonic acid in the HF-extracted polysaccharide may be an indication that the polysaccharide is a teichuronic acid. Molnar et al (26) had earlier indicated that B. anthracis lacks teichoic acid, and the structure of HF-extracted polysaccharide seems to lend support to their observation.

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Aim: To quantitate the binding of soybean agglutinin to Bacillus anthracis.

Materials: ^{3}H-acetic anhydride, SBA, saturated sodium acetate, dialysis tubing, lyophilized samples of B. anthracis Sterne cells, heat-treated cells, trypsinized cells; Δ-Sterne cells, Δ-Sterne spores, cell walls and 4229 cells, N-acetyl-galactosamine (GalNAc) (10 mg/ml PBS).

Method: 1 ml of a 50% saturated sodium acetate solution was added to 5 mg SBA (EY-Labs). This mixture was then transferred to a dialyses bag along with ^{3}H-acetic anhydride. The reaction is instantaneous. (The presence of sodium acetate enhances N-acetylation). The bag was tied off and dialysed against 0.1N NaCl for 24 hr. The final dialysis was in PBS. The retentate was analyzed for radioactivity as well as SBA activity before using it in this procedure.

Cell/spore suspensions were made in PBS (2 mg/ml). 500 ul of each sample were incubated (1 hr. 37^{o}C) with 100 ul of the ^{3}H-SBA. The controls contained 500 ul cell suspension + 100 ul PBS and 500 ul PBS + 100 ul ^{3}H-SBA. All samples were incubated for 1 hr at 37^{o}C and then centrifuged. 100 ul of the supernatant were counted, and the bound SBA subsequently calculated. Since SBA has a specificity for the sugar GalNAc, it was thought opportune to include this in an independent experiment as the competitive inhibitor. 500 ul of the organism was added to 100 ul of the ^{3}H-SBA containing 500 ul GalNAc and processed as before.

Results:

Table 7 shows that out of the three strains of B. anthracis tested the order of binding of SBA was 4229 > Δ-Sterne cells. The cell walls retained maximum SBA and the spores were seen to be more radioactive than the cells. But when the cells were heat treated or trypsinized before exposing to SBA, the binding seemed to be significantly increased. The latter observation was not easily discernible and could not have been quantitated with the previous fluorescence antibody studies.

When GalNAc was included in the mixtures, there was insignificant binding of the SBA.

TABLE 7

Binding of Soybean Agglutinin to *Bacillus anthracis*

Test	SBA Bound (ug)
Bacillus anthracis Sterne cells	4.8
Bacillus anthracis Sterne cells heat-treated	17.0
Bacillus anthracis Sterne cells trypsinized	10.9
Bacillus anthracis ΔSterne cell	8.9
Bacillus anthracis ΔSterne spores	16.2
Bacillus anthracis ΔSterne cell walls	31.1
Bacillus anthracis ATCC 4229 cells	10.8

In a total volume of 600 ul were 150 ug [^{3}H]SBA and 1000 ug intact cells, spores or cell walls. All suspensions were in 40 mM sodium phosphate, 150 mM sodium chloride (pH 7.2). When 10 mg/ml GalNAc was included in the mixtures, there was no detectable binding of SBA. The specific activity of [^{3}H]SBA was 850 CPM/ug.

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EFFECT OF SODIUM PERIODATE TREATMENT ON THE CELL-SBA INTERACTIONS

PROTOCOL:

B.anthracis Sterne cells were grown overnight in the R-Medium. They were harvested and washed 2x with PBS. One batch of cells was treated with 0.2M sodium periodate (overnight, 4°C, covered) and the other was left in PBS. After 18 hrs, the cells were washed 3x with PBS and subjected to the cell-SBA aggregation test on the Boerner plates.

RESULTS:

The untreated, control cells,aggregated strongly in the presence of SBA. As low as 15.62 ug/ml of the lectin was sufficient to bring about discernible aggregation. The sodium-periodate modified cells did not show any aggregation. SBA concentrations as high as 500ug/ml failed to bring about any signs of aggregation.

The above results lend support to the fact that there are galactose residues on the surfaces of B.anthracis cells and that sodium-periodate does indeed remove or modify these residues on the cells. Thus making them incapable of interaction with the lectin SBA.

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Leighton-Doi Anthrax Spore Media

(W.F. Burke Jr., J. Gen. Micro. (1982) 128:1591-1597)

INGREDIENTS:	Grams/liter	Grams/500ml	mg/500ml
1) Nutrient Broth	16	8	8000
2) Glucose	0.9	0.45	450
3) KCl	1.86	0.93	930
4) $CaCl_2.2H_2O$	0.294	0.147	147
or $CaCl_2$	0.22	0.11	110
5) $FeSO_4.7H_2O$	0.00278	0.00139	1.39
or $FeSO_4$	0.00152	0.00076	0.76
6) $MnSO_4.H_2O$	0.00169	0.00085	0.85
or $MnSO_4$	0.00151	0.00076	0.76
7) $MgSO_4.7H_2O$	0.0246	0.0123	12.3
or $MgSO_4$	0.012037	0.00601	6.01

pH 6.8 is Optimal.

Note:-

- Make up the salts separate from glucose and nutrient broth. Autoclave. Combine when cool to avoid precipitation.

- Package no more than 10% of flask volume per flask in order to insure adequate oxygenation.

PREPARATION OF "R" MEDIUM STOCK SOLUTIONS

Stock Solution "AA" (Amino Acids), 40X

To prepare 40X stock solution of amino acids, salts, and nucleotide bases, add the indicated amounts of the components listed below to approximately 400 ml distilled wate. (Milli-Q) while stirring. Adjust final volume to 500 ml. Do not adjust pH.

Component	Amount g/500 ml	g/250 ml
L-leucine	4.60	2.30
L-histidine	1.08	0.54
L-proline	0.86	0.43
L-tryptophan	0.72	0.36
L-phenylalanine	2.52	1.26
Glycine	1.30	0.65
L-lysine	4.60	2.35
L-arginine	2.52	1.26
L-methionine	1.44	0.72
L-isoleucine	3.40	1.70
L-threonine	2.40	1.20
L-serine	4.68	2.34
L-valine	3.46	1.73
$MnSO_4$	0.017	0.0085
$CaCl_2.2H_2O$	0.147	0.070
$MgSO_4.7H_2O$	0.197	0.098
Adenine sulfate	0.042	0.021
Uracil	0.028	0.014
Thiamine HCl	0.020	0.010
L-Na glutamate	12.24	6.12
L-aspartic acid	3.68	1.84

Divide into 25 ml amounts and store in 50 ml polypropylene screw-top tubes at -10 to -20° C.

Stock solution "Dex-Phos", 40X

Dextrose	50 g
K_2HPO_4	60 g

Add distilled water to 500 ml, divide into 25 ml amounts, store frozen in 50 ml polypropylene screw-top tubes.

Stock solution "Tyrosine", 1000X

Tyrosine 2.88 g in 20 ml of 1 N NaOH. Store frozen.

Stock Solution "Cystine", 1000X

L-cystine 0.5 g in 20 ml of 1 N HCl. Store frozen.

R-medium preparation

Add a tube of "AA" and a tube of "Dex-Phos" to approximately 500 ml distilled water in a 1 liter beaker while stirring.

Add 1.0 ml each of "Cystine" and "Tyrosine" solutions, and, where applicable, add 7.88 g Tris-HCl (Mol. Wt. 157.6). Adjust pH to 7.5. Add 8.0 g sodium bicarbonate and adjust volume to 1 liter. Filter sterilize. Dispense into sterile screw-top flasks. Medium volume should be 1/4 to 1/3 of the flask volume and the screw-top caps should be tightened.

SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS

Materials:-

1. Acrylamide-bisacrylamide 44% : 0.8% solution
2. 1.5 M Tris buffer, pH 8.8
3. 10% SDS
4. Acrylamide-bisacrylamide 30% : 0.8% solution
5. 0.5 M Tris buffer, pH. 6.8
6. Ammonium persulfate, 10 mg/ml
7. TEMED
8. Saturated butanol (In Tris buffer, pH. 8.8)
9. Stain: 1.3g Coomassie Blue, 600 ml methanol, 600 ml H_2O and 133 ml acetic acid.
10. Destain: 10% acetic acid + 15% methanol.
11. Electrode Buffer: 3.03 g Tris base (0.025M), 14.27g glycine (0.19M), 1.0g SDS (0.1%), 1000 ml H_2O.
12. Sample Buffer: 2 ml Tris buffer (0.5M, pH 6.8), 4 ml, 10% SDS, 1 ml 2-mercaptoethanol, 2 ml glycerol, 0.2 ml, 0.1% bromophenol blue, 0.8 ml H_2O.

Method:-

Depending on the percentage of running gel desired, the solutions can be made according to the proportions given in the Table below. The Acrylamide-bisacrylamide solutions are stable for a month in the refrigerator. The ammonium-persulfate should be prepared immediately before use.

TEMED is stable at refrigerator temperatures and is the component responsible for crosslinking the acrylamide.

RUNNING OR SEPARATING GELS

	Quantities in ML					
	6%	8%	10%	11%	12%	14%
Acrylamide:Bisacrylamide						
44:0.8	3.5	4.5	5.68	6.25	6.82	7.95
Tris-Hcl buffer 1.5M, pH 8.8	6.25	6.25	6.25	6.25	6.25	6.25
SDS, 10%	0.5	0.5	0.5	0.5	0.5	0.5
Deionized water	14.25	13.07	11.89	11.32	10.75	9.62
Ammonium persulfate	0.63	0.63	0.63	0.63	0.63	0.63
TEMED (Kodak)	0.05	0.05	0.05	0.05	0.05	0.05

Double the above volumes when using the large Protean Cell from BioRad

STACKING GEL (3%)

	Quantities in ML
Acrylamide:Bisacrylamide 30:0.8	3.0
Tris buffer 0.5 M, pH 6.8	5.0
SDS, 10%	0.2
Deionized water	11.28
Ammonium persulfate 1%	0.48
TEMED (Kodak)	0.04 (40 ul)

Note: It is appropriate to run the stacking gel at 25 mA, and the separating gel at 35 mA It takes 3 to 4 hours to run a 10 cm gel.

After pouring the separating gels it is recommended to place 0.5 ml saturated butanol on the top, in order to make a very uniform and straight edge. The butanol is then rinsed off with distilled-deionized water and the stacking gel poured on top. Desired comb is used to make appropriate wells.

The proteins to be tested and the standard molecular weight markers are diluted 1:1 in sample buffer boiled for 4 min and placed in respective wells and electrophoresed. When the samples have travelled down about 10 cm, the system is shut off, gel is removed from the glass plates and either fixed immediately or stained. The gels can be preserved by drying on a gel-dryer.

Uranyl Acetate/Lead Citrate Staining

The uranyl acetate stain was made as a 2% solution in 50% methanol.

For lead-citrate, Reynold's method was employed. 1.33g of lead-nitrate were combined with 1.76g of sodium-citrate and added to 30 ml of boiled deionized water. The mixture was shaken vigorously and continuously for 1 min. The mixture was shaken intermittently for the next 30 min and finally added to 8 ml of IN NaOH. The total volume was then brought upto 50 ml.

The grids were stained for 30 seconds with uranyl acetate and rinsed with distilled-H_2O, followed by lead-citrate for another 30 seconds and rinsed throughly again. The grids were then ready for viewing.

PREPARATION OF O-STEAROYL-POLYSACCHARIDE

(Hammerling, U., & Westphal, O. (1967) Eur. J. Biochem. 1:40-50)

Procedure:-

1) Weigh 50 mg pCHO in ampoule (dehydrate).

2) Dissolve pCHO in 4 ml anhydrous dimethyl formamide (DMF) at 50°C.

3) Add 0.6 ml anhydrous pyridine and 10 mg stearoyl chloride dissolved in 0.1 ml DMF.

4) Seal the ampoule (include a small stir bar before sealing).

5) Stir the reaction mixture at RT for 3 days.

6) Dilute the reaction mixture with 0.5 ml distilled-deionized water.

7) Pour reaction mixture into 25 ml alcohol.

8) Let stand for several hours at 4°C.

9) Collect precipitate by centrifugation, wash with absolute ethanol, let dry at RT, and dissolve in 5 ml dd H_2O.

10) Freeze dry the sample.

(Note:- 25 mg of the pCHO was processed in lab. 15 mg of the o-stearoyl-pCHO was obtained).